ANALYTICAL MICROSCOPY

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ITS AIMS AND METHODS

BY

T. E. WALLIS

B.Sc. (Lond.), F.I.C., Ph.C.

MEMBER OF THE SOCIETY OF PUBLIC ANALYSTS
LECTURER IN BOTANY TO THE PHARMACEUTICAL SOCIETY OF GREAT BRITAIN

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PREFACE

The Public Analyst, the Analyst in general consulting practice and the Pharmacist frequently meet with problems which can only be satisfactorily and completely solved by a skilled use of microscopical methods.

The majority of these problems are neither bacteriological nor physiological and there is, at the present time, no reliable book to which the analyst can refer for information with respect to the methods employed for the microscopical examination of the numerous miscellaneous substances that are brought in for analysis. Neither is there any existing guide that will indicate the best standard works in which information about such matters may be found. This information is scattered in many volumes, and the necessity for reporting in precise terms makes it essential, for the successful conduct of a general and consulting analytical practice, to be able to find the exact details required.

The following chapters contain an account of the methods which the author has found most generally serviceable during several years' practical experience of such analytical work. The sources of detailed information upon the subjects dealt with are indicated by reference to the names of the various authors concerned and the titles of their publications.

No attempt has been made to provide an exhaustive treatment of all the microscopical work that may be encountered; the book contains no bacteriology or clinical microscopy, nor does it provide an atlas of drawings. Methods for the systematic study of typical vegetable

structures by sections and in other ways have not been included; an adequate exposition of this type of work can be found in Greenish's *Microscopical Examination of Foods and Drugs* and in Winton's *Microscopy of Vegetable Foods.*

The aim has been to open up new ground by studying the methods of approaching microscopical problems and to limit the application of these methods to investigations which cannot be satisfactorily completed without the use of the microscope.

With the exception of figures 1, 6, 22, and 23 the illustrations, many of which are original, have been specially drawn for this book, and of these all except figures 8, 9, 18, 22, 37 and the five plates were first published in the pages of the *Pharmaceutical Journal.* I am indebted to the Editor of that Journal for placing these blocks at my disposal for use in the printing of this book. For Fig. 1, my thanks are due to Messrs. C. Baker, of 244, High Holborn, and for Fig. 6, to Messrs. Watson & Sons, of 313, High Holborn, London.

My wife has drawn for me figures 2 to 5, 7, 11, 15, 16, 21, 22, 26, 27, 36, 39, 43, 45, and the figures on plates A, B, D, and E. For the remaining figures I am responsible.

This book had its origin in a series of articles upon "Analytical Microscopy," which were published in the *Pharmaceutical Journal* between March, 1920, and January, 1921. Many readers have expressed a wish to see these published in book form, so that I have submitted them to a thorough revision and added a large amount of new matter in the hope that they may prove useful in book form to a still wider circle of readers.

T. E. WALLIS.

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CHAPTER I

INTRODUCTION

Limitations of the Microscope. The microscope often yields information of first-rate importance in analytical investigations, frequently such as cannot be discovered by any other method. For this reason an extended and thorough acquaintance with microscopic structures and methods of working is of the greatest value to analysts. One must, however, remember that this information is always obtained by increasing one's power of sight, and hence microscopy is unable to give any effective assistance where quantities visible to the unaided eye can be made to yield the same results. The practical utility of microscopical methods is therefore limited in extent, and the results obtained are generally considered in connection with important factors obtained by other methods, so that one must be on one's guard against expecting too much from a method of working which is sometimes brought into disrepute when it is found that the performance does not meet the expectation.

Subjects for Microscopical Investigation. In the hands of the analyst it is in the examination of the following groups of substances that the microscope finds its most important application :—

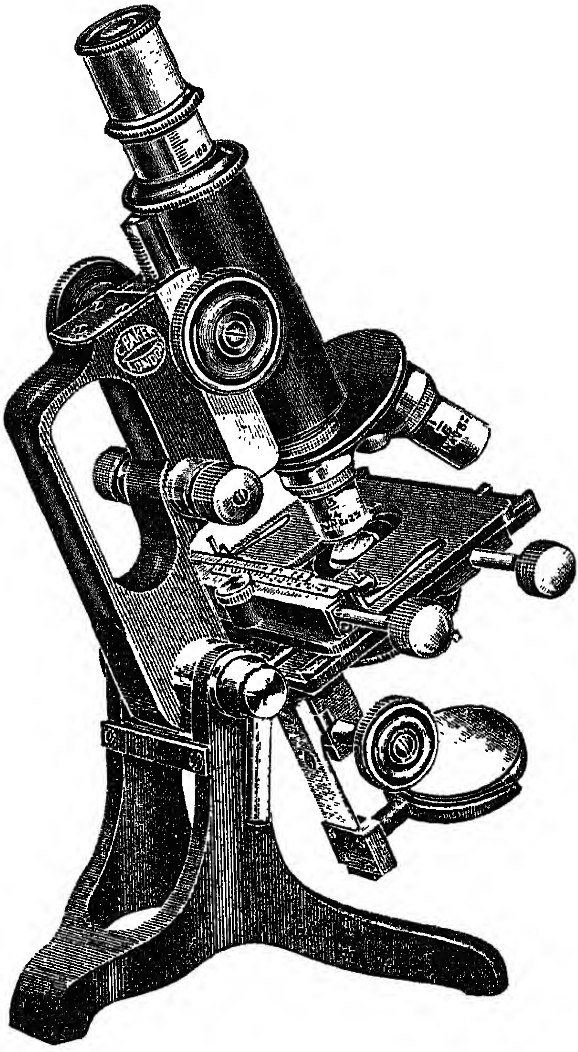
1. Foodstuffs, such as flours, meals and sugars.
2. Drugs and spices, especially when powdered.
3. Galenicals, such as extracts, pills, powders, tablets, etc.
4. Feeding cakes, farm seeds, etc.
5. Jams and preserves.

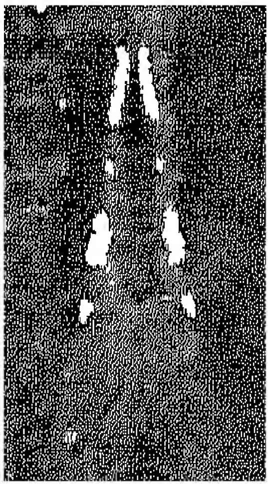
6. Deposits from water (including the examination of plankton), urine, sewage, etc.
7. Fibres used in the manufacture of fabrics, surgical dressings and paper.

Nature of the Work. It is evident that a very varied knowledge of biological structures and much miscellaneous information are required by those who wish to become expert in the use of the microscope as an aid to analysis, and numerous type subjects covering the wide range of substances indicated must be systematically studied before one can hope to use the instrument successfully. Having obtained a general acquaintance with the methods of attacking the various problems likely to be presented, one is able to decide upon and carry out the preliminary studies necessary for any special investigation. In a general sense some work with authentic material is desirable before satisfactory deductions can be drawn; this work, however, is rendered much less laborious by making use of published drawings and descriptions, which frequently enable one who has had the necessary introductory practice to detect rapidly any foreign substance, and then, by comparison with material of known origin, the conclusions may be confirmed. When using atlases and drawings it is always desirable to adopt this method of confirmation in cases where foreign matter, whose nature is not immediately recognized, has been found, since from time to time one may come across structures which are normally present but have escaped description.

In addition to these qualitative results it is possible in certain cases to make accurate quantitative determinations of the proportions of substances in mixtures of materials such as cannot be separated by chemical processes.

The Microscope. The instrument to be used for so great a variety of work needs to be carefully selected; it must be equipped with lenses covering a wide range of magnification, and should be provided with a substage condenser and a polariscope. A good stand with a large





to accommodate the microscope, a lamp, material for drawing, and a number of bottles containing stains and mounting media. Such a table will be about 4 ft. by 2 ft. in area, and not more than 30 in. in height; one that is of the pedestal form and provided with drawers can be used as a store-place for all kinds of microscope accessories, such as glass slides, dishes, needles, drawing apparatus, etc. If the laboratory is so situated that it is possible to use daylight, the table should be placed near a window and preferably about two or three feet away from it, an arrangement which protects the table and anything placed upon it from injury when the window is opened, and from risk of disturbance when raising or lowering the dark-coloured blind with which the window should be provided.

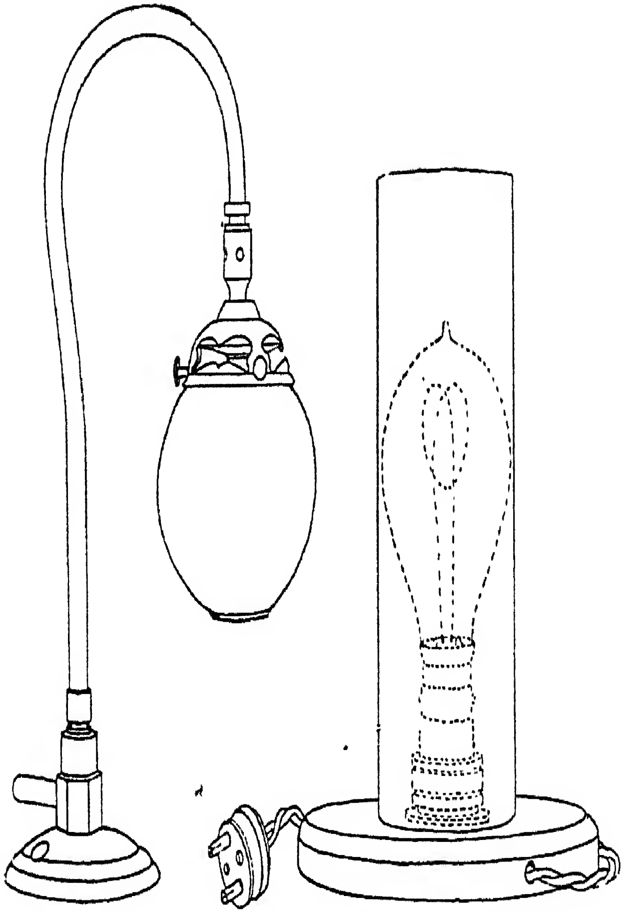
The special table should be placed near to the preparation bench, either in the same room or in an adjacent one. The microscope itself should be kept ready for use at a moment's notice, and the lenses most commonly needed should always be in position upon the instrument, which may be protected from dust and fumes by covering it with a bell-glass. For cleaning slides and cover-glasses it is a good plan to provide two glass or earthenware pots filled with water—a smaller one into which the cover-glasses are pushed when finished with and a larger one into which one may drop the glass slides. The use in this way of a separate vessel for the cover-glasses will result in saving many which would be broken if slides and covers were put into the same pot. Two small conical flasks of about 100 c.c. capacity, fitted with indiarubber corks, through each of which passes a piece of quill glass tubing drawn out to a fine jet, are useful to keep a supply of alcohol and distilled water ready to hand upon the table. The lamp and bull's-eye collecting lens should also be kept in position, so that, on lighting the lamp, everything is prepared for immediate use.

Illumination. The provision of suitable illumination is worthy of special attention. Daylight is satisfactory for most ordinary analytical work, yet the difficulty of

securing a suitable position for the microscope table and the variations produced by the movements of clouds make it necessary and often preferable to use artificial light. An oil lamp with a half-inch wick is the most convenient and generally useful illuminant. For those who prefer gas, an incandescent burner may be used; but the mesh of the mantle is apt to produce a confusing effect when sharply focussed for critical work. This difficulty is overcome for most ordinary work by using a ground glass shade (Fig. 2). The ground surface of the glass then forms the source of illumination, and can be focussed by a bull's-eye and substage condenser in the ordinary way.

The use of electric lamps, both of the filament and arc types, is strongly advocated by some workers, chiefly on account of the greater convenience in cleaning and lighting, and also, in certain cases, because a light of much greater intensity is obtainable; but the advantages do not appear to be so great as to make the use of electricity preferable under all circumstances. Most filament lamps are unsuitable for use with a substage condenser, and one generally has to modify or cut down the light from an electric lamp by the use of screens and condensers before it will yield satisfactory results. A very useful and evenly distributed light for regular use is obtained by covering an ordinary filament lamp with a closely fitting cylinder of parchment paper, as shown in the accompanying figure (Fig. 2). The cylinder is made of a sheet of the paper, secured by a pin, and can be easily replaced when dirty or discoloured by the heat. A further improvement is obtained by enclosing the parchment paper cylinder in one of black paper from which a circular window, about 2 in. in diameter, has been cut. This adds greatly to the comfort of working, and does not reduce the light received by the microscope.

The "Fullolite" lamp, in which an opal bulb replaces the plain glass bulb of an ordinary filament lamp, is an excellent source of illumination for regular use; it should, however, be screened by an opaque cover so as



IG. 2.—Incandescent Gas Lamp and Electric Filament]

Fig. 3.—Paraffin Oil Lamp with Iron Chimney and Metal Shade. Also a "Bull's-eye" Condenser for use with the Lamp.

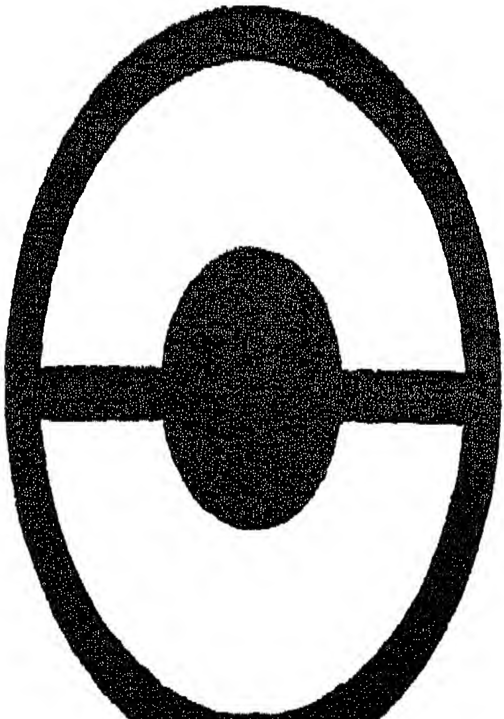
ated, the distance between the lamp and mirror must be less for lower-power objectives than for the higher powers. With quite low powers, it is best to use a special low-power substage condenser or to remove the front lens of the ordinary condenser.

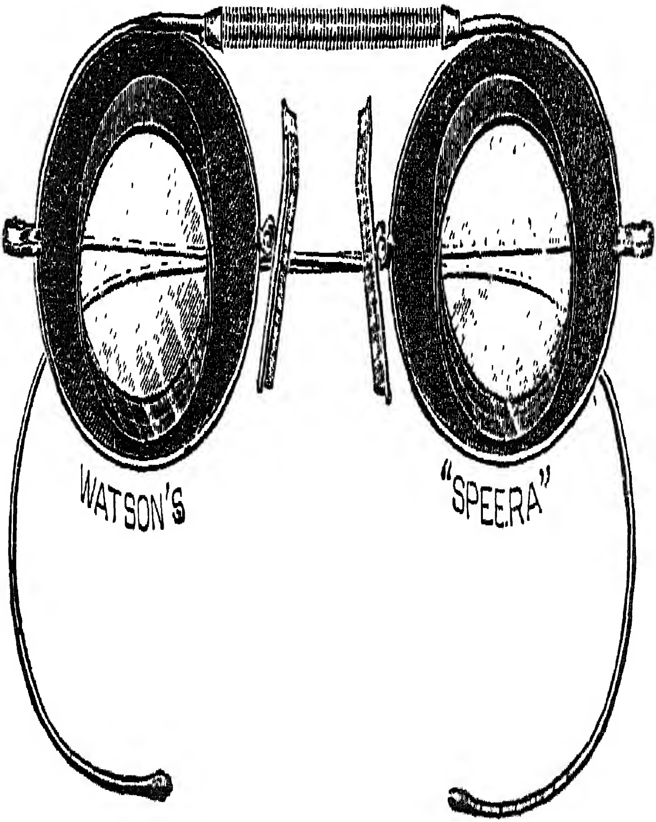
Critical Illumination. To obtain critical illumination, the condenser is first centred by making a small ink dot in the centre of the top lens of the Abbé combination, focussing

this spot with a low-power objective, and bringing it into the centre of the field by adjusting the centering screws of the substage. The ink spot is cleaned off and a slide is placed on the stage, the object is focussed, and the image of the lamp flame is brought sharply into focus by racking the substage up or down as needed. The bull's-eye collecting lens is then placed near the flame, towards which its flat face is turned, and the enlarged image of the flame is focussed upon the under-surface of the substage condenser. This is best done by holding a small piece of white paper against the under-surface of the Abbé condenser, and moving the bull's-eye lens until the flame-image is sharply focussed upon the paper, as seen reflected in the plane mirror. A little adjustment of the bull's-eye lens, up or down or sideways, will now produce a lemon-shaped evenly-illuminated area in the field of view of the microscope, and one has the best illumination available—i.e. critical illumination.

Dark-ground Illumination. Dark-ground illumination is in certain cases of the greatest value, as, for example, in the examination of crystals, diatoms, and some insect structures. For this purpose one uses a central opaque stop, which is conveniently made of blackened cardboard and is supported in a carrier immediately below the substage condenser. A larger stop is needed with a low-power condenser than with one of higher power (see Fig. 4). The condenser is adjusted in the way usual for ordinary illumination; the stop is then placed in the carrier, which is swung into position under the condenser, when the object is seen as a self-luminous body upon a dark background. If the ground is not properly darkened, a slight adjustment of the focus of the condenser will remedy the fault.

By the use of this method, structures which have nearly the same refractive index as the fluid in which they are mounted can be made easily visible, and fine striations become evident. The best results are obtained with




WATSON'S "SPEERA"

and a supply of small dishes in the form of hollowed glass blocks with covers will be found indispensable for handling and staining sections. Drawings of these various articles are shown in Fig. 5.

For naked-eye dissections, a pocket lens will be needed, or one may use the "Speera" Binocular Magnifier (Fig. 6) mounted like a pair of spectacles and made by Messrs. Watson & Sons of Holborn, London, W.C.1, which will also be found invaluable for examining quantities of material such as chicken foods and cattle cakes.

CHAPTER II

SIMPLE METHODS OF PRELIMINARY TREATMENT

Necessity for Preliminary Operations. In a few instances only is it safe to assume that an examination of a substance without preliminary treatment will enable the microscopist to pronounce an opinion upon its composition. One might imagine that materials like starches, spores, or pollens could be sufficiently examined by the very simple method of mounting them in water or dilute glycerin and carefully studying the preparations. This operation should not be neglected; yet, excepting in those cases where an entirely different substance has been either purposely or inadvertently substituted, it is probable that the worker would overlook small quantities of an inconspicuous admixture. It is for this reason that one should devise some preliminary operations whose aim is to sort the material into various components, in each of which certain types of structures may be expected to become concentrated.

There are many such methods which one is constantly using. Some of them are comparatively simple, such as sifting, kneading, sedimentation, centrifugation, and elutriation. Other methods are much more complex and involve a more drastic treatment of the material; such are preparation of crude fibre by digestion with acid and alkali, wet-washing by heating with strong sulphuric acid, treatment with a solvent and collection of the residue. Another group of operations aims at bringing certain constituents into prominence by colour reactions or by the use of chemical reagents whose action results in the formation of

products having a structure easily recognized under the microscope. To obtain such effects one may subject the material to various staining processes or to the action of certain chemicals either upon the slide, which is kept continually under observation on the microscope stage, or in test tubes as a preliminary to the microscopical examination.

Sifting. The simpler processes may be considered first. Sifting is useful in a large number of instances. For example, weed seeds are commonly smaller than those among which they occur, and can be separated and concentrated by the use of a sieve having a suitable mesh. The seeds so removed can be examined under a low power of the microscope without further preparation, and identified by comparison with standard specimens. In this way one can discover the seeds of harmful weeds, seeds of farm plants, seeds of poisonous plants, and seeds of weeds commonly found among farm seeds. Valuable assistance in such work may be obtained by reference to good photographs of seeds at known magnifications, such as are to be found in a useful little book entitled *Impurities of Agricultural Seed*, by Parkinson and Smith. The Board of Agriculture leaflets Nos. 326, 251, and 112 contain useful information upon the same subject.

The chief points to be noted in examining seeds are the size, shape, colour, hilum, and surface markings, such as furrows, spines, projections, and reticulations. The wheat "screenings" used in making mixtures for poultry feeding contain large numbers of these weed seeds, and it is useful to be able to identify them when inquiries are made about such mixtures. Since much wheat comes from abroad, many of the seeds are those of foreign weed plants, and these, as well as seeds of indigenous plants, are figured in the work mentioned above.

Spices for Cattle and Poultry. Sifting is also applicable to foods, meals, and spices for poultry and cattle. In dealing with such mixtures of smaller and larger par-

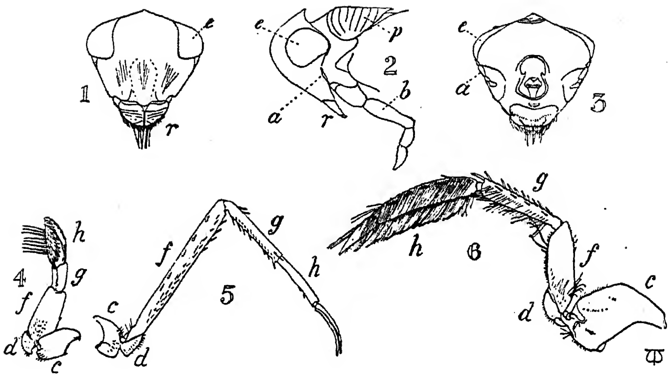


FIG. 8.—*Corixa mercenaria.* Sav.

an hour; next boil them for a few minutes in glacial acetic acid in a test-tube and allow them to soak in the hot liquid for about 20 minutes. Pour out the contents of the tube into a dish, take out the insect parts on the point of a needle, place them upon a glass slide, add a drop of glycerine and cover them with a cover-glass.

possibly in very small amount, but characteristic either of the substance itself or of certain admixtures which might otherwise be overlooked. (See hairs of pepper, Fig. 38, p. 112.)

Kneading. Kneading a substance in a calico or muslin bag under water or some other specially selected liquid is a process closely akin to sifting, and is particularly applicable to flours and other starchy powders. The turbid liquid produced by the kneading is allowed to settle, and the supernatant fluid is decanted. The deposit is washed two or three times with water by decantation, after which the starch is filtered out and allowed to drain until partly dried. The filter is then removed from the funnel and spread out, so that the starch may dry by exposure to the air. The air-dry product is kept for a while spread out on the filter paper in a warm place, and is finally removed from the paper, powdered, and stored in a specimen tube for future use.

Flours made from damaged grain will yield a starch showing numerous grains partially eaten away by enzyme action, and frequently also fungal spores will be found intermixed. A detailed examination of the starch gives information as to the constituents of the flour, and by making counts of the various types of starch grains present a very good estimate of the proportions of such constituents can be obtained. This problem will be more fully discussed under the heading of Quantitative Microscopy.

Drawings of some of the commoner types of starch grains will be found in Figs. 10, 17 and 18, where the starches are represented at a uniform magnification of 400 diameters.

Wheat Flour containing Barley and Maize. After the kneading of a starchy powder is completed the bag is opened out and stretched over a watch glass of suitable size, and the residue removed by a flexible spatula. In the case of the glutinous substance obtained from flours, acetic acid may be added to the residue in a flask. It is allowed to soak for a while to dissolve the bulk of the gluten, and is

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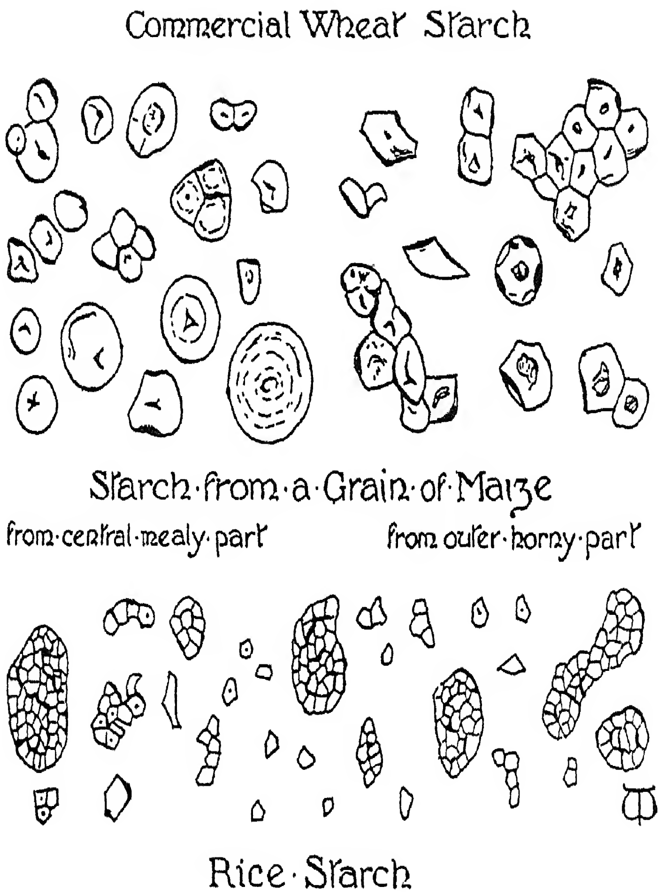


FIG. 10.—Wheat, Maize, and Rice Starches.

All × 400.

then again poured into the kneading cloth arranged so as to line the inside of an evaporating dish. The cloth is tied up, and the kneading proceeded with until the water leaves the bag clear. The residue is removed as before and reserved for microscopical examination. In this way one obtains a material closely resembling crude fibre; but, from some points of view, in a better condition, because it has been subjected to a much less vigorous treatment. Here will be found concentrated the characteristic hairs, if any, of the cereal, and the various structures belonging to the pericarp and seed coats; also the cells of the aleurone layer and of the endosperm.

The addition to wheat flour of small amounts, up to about 5 per cent., of maize or barley flour is very difficult to detect by an examination of the flour without preliminary treatment. The best procedure to adopt is to sift the flour through a No. 80 sieve and examine the portion that fails to pass. This fraction contains the harder part of the maize and the more chaffy portions of the barley, and should be well kneaded in a cloth first with water, and then with diluted acetic acid. If the flour contains maize a microscopical examination of the residue in the cloth will now show small angular masses consisting of the harder part of the maize grain containing the closely-packed characteristic starch. If barley is present it can be definitely identified by finding portions of the characteristic outer epidermis of the flowering glume, although it is impossible, owing to the similarity between the starches of wheat and barley, to detect barley with certainty by a simple qualitative examination of the starch. These details are described and figured by Greenish and Collin in their *Anatomical Atlas of Vegetable Powders.*

Flour infested by Mites and Insects. Mites, "worms" or grubs, moths and beetles present in flour in small numbers such as would escape detection by a simple inspection, may be concentrated in a small quantity of the flour by sifting through a No. 80 sieve. These

creatures are then easily discovered by examining with a lens the residue left on the sieve. The most convenient way of looking for mites is to transfer the material to a glass tube, and to scrutinize the contents of the tube with a hand lens in a good light. The suspected particles are removed on the point of a knife or a needle, and mounted in chloral phenol (equal parts by weight of chloral hydrate and phenol crystals warmed together until liquefied), when the mites become cleared, and can be identified (see Fig. 11).

The four following mites have been reported as occurring in grain, flour and fodder: *Tyroglyphus siro*, Linn., the common cheese-mite; *Tyroglyphus longior*, Gervais, a smaller mite with a body longer in proportion, and more oblong in shape; *Aleurobius farinæ*, Koch, the flour mite, which may be recognized by the curious outgrowths upon the front legs of the male; and *Glyciphagus spinipes*, Koch, which has long feathered hairs. In addition to these one also finds the carnivorous mite *Cheyletus eruditus*, Latr., which feeds upon the other weaker and smaller mites. A full description of these and other mites is given by A. D. Michael in his monograph on the *British Tyroglyphidæ*.

The occurrence of acarids in flour has been recently investigated by Newstead and Morris (1920), who give their conclusions as follows: "In the earlier Report by Newstead and Duvall (1918), the only acarid recorded from flour was *Aleurobius farinæ*. A more extended search has, however, revealed the presence of the following additional species: *Tyroglyphus longior*, Gervais; *Histiogaster entomophagus*, Laboulbene; *Glyciphagus fuscus*, Oudemans; and *Cheyletus eruditus*, Schrank. Of these four species the first two are of primary importance as they are capable of causing considerable damage to stored flour; but they appear to be much more localized in their distribution, and therefore less destructive generally, than *Aleurobius farinæ*. It appears to us that the injury caused by *Glyciphagus fuscus* is negligible, as in captivity the

females, although amply supplied with food, were markedly less prolific than any of the other Tyroglyphids under

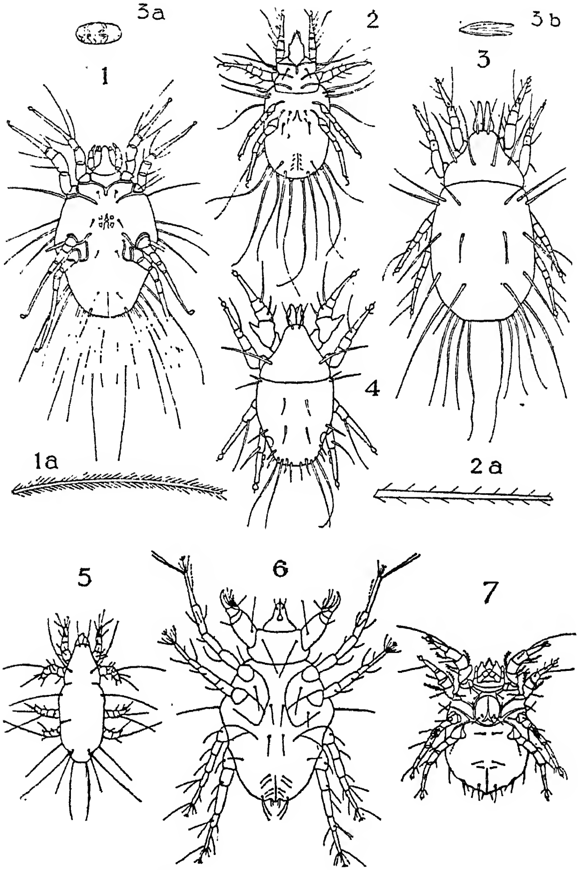


FIG. 11.—Mites found in Flour and Fodder.

(1) *Glyciphagus spinipes*, Koch. (1*a*) Hair of Same to Show Feathering. (2) *Tyroglyphus longior*, Gervais. (2*a*) Hair of Same to Show Feathering. (3) *Tyroglyphus siro*, Linn. (3*a*) Egg and (3*b*) Empty Case of the same. (4) *Aleurobius farinæ*, Koch. (5) *Histiogaster entomophagus*, Laboulbene. (6) *Cheyletus eruditus*, Schrank. (7) *Glyciphagus fuscus*, Oudemans. Mites and Eggs all × 40. Hairs × 200. (1), (2), (3) and (4) all after Michael. (5), (6) and (7) after Newstead and Morris.

observation. The predaceous *Cheyletus eruditus* occurred sporadically, but its presence did not appreciably check the ravages of those Tyroglyphids with which it was associated."

The grubs most commonly found in flour are the larvæ of the moths *Ephestia kühniella*, Zeller, and *Corcyra cephalonica*, Stainton. The larvæ of the former moth have a pale pink tint, while those of the latter are dull whitish. They spin silken threads which entangle their droppings (consisting of starch) into loose masses. The droppings may be found when the larvæ themselves have been removed by sifting, and the fact that the flour is infested by the moth is thus revealed.

Borkhausenia (also known as *Acompsia* or *Œcophora*) *pseudospretella* is a third moth, whose larva is frequently found in flour and a great variety of other commodities; it is, in fact, one of the commonest household pests. The drawings in Fig. 12 show the characteristic features and markings of these three moths, all of which more or less resemble the common clothes moth in external appearance. Details of the head with its palps and of the venation of the wings are given in Fig. 13 for the moth *Borkhausenia pseudospretella* Stt., to show the minute particulars which are necessary for the identification of such moths. The corresponding characters for other moths may be found in the works of Stainton, Durrant and Beveridge and of Meyrick quoted in the General Bibliography.

The wings of these moths are prepared for the examination of the neuration by taking them from the insect, placing them upon a glass slide and removing the scales by gently brushing them with a camel-hair pencil dipped in alcohol. For purposes of reference, the wings may be allowed to dry on the slide and are then mounted as dry objects by applying a cover-glass and fastening it down by small strips of gummed paper.

It is, of course, the caterpillars which do so much damage,

and drawings of two of these, with the pupa of one of them, and an enlarged drawing of the head of a third, to show the biting jaws, are given in Fig. 14.

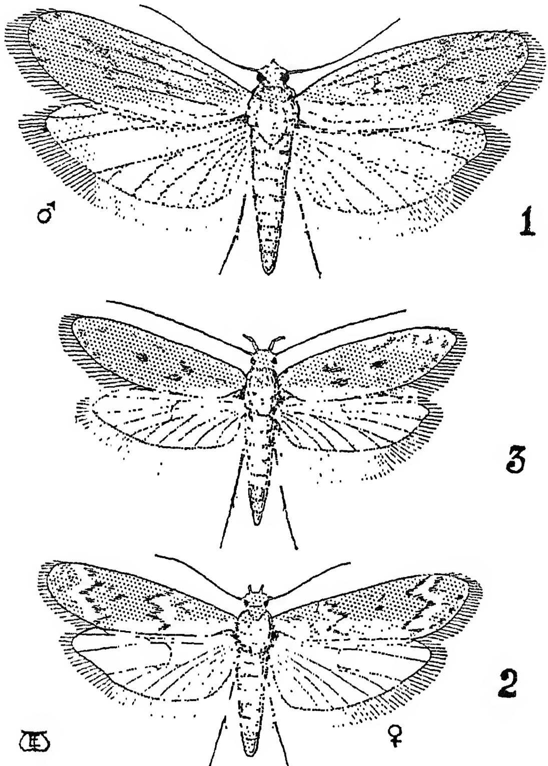
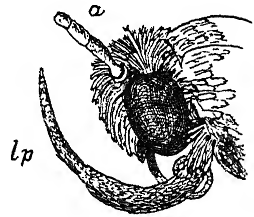


FIG. 12.—Moths whose Larvæ attack Drugs.
(1) *Corcyra cephalonica*, Stt. (2) *Ephestia kühniella*, Zeller. (3) *Borkhausenia pseudospretella*, Stt. All × 3.

Three beetles are frequently found; these are the grain weevils, *Calandra granaria*, Linn., and *C. oryzæ*, Fab., and one of the *Ptinidæ*, viz., *Sitodrepa panicea*, Thoms., which is closely allied to the "death-tick" or furniture beetle,

Anobium domesticum, Fourc., and is sometimes known as *Anobium paniceum*, Fabricius. This same beetle is also often found in large numbers in parcels of drugs, and for



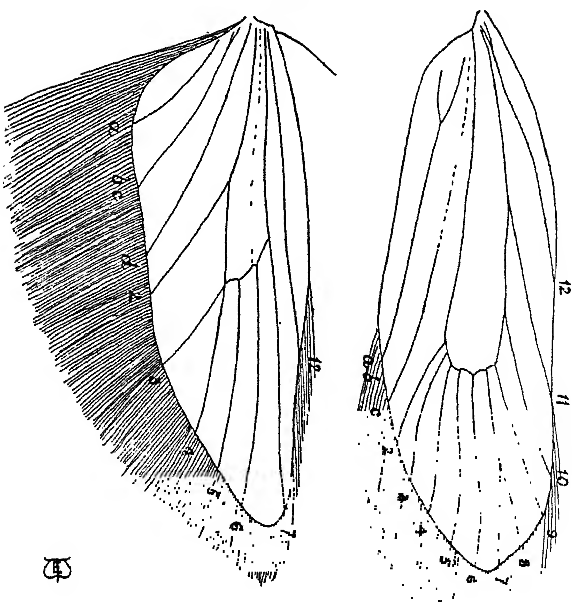


Fig. 13.—*Borkhausenia pseudospretella*, Stainton.

Head × 20. *a*, antenna; *lp*, labial palp; the rounded darkly-shaded area is the eye and the curved organ projecting from the head beneath is the proboscis. Wings showing the neuration × 8.

this reason has received the name "drug-room beetle" (see Greenish and Braithwaite, *Pharm. Journ.* 1910 [4] 31, 580).

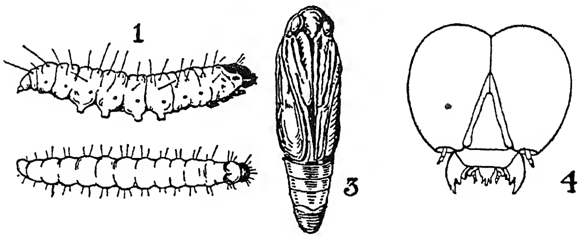


FIG. 14.

(1) Larva of *Ephestia kühniella*, Zeller, × 2. (2) Larva of *Corcyra cephalonica*, Stainton, × 2. (3) Pupa of *Ephestia kühniella*, Zeller, × 2. (4) Head of the larva of *Borkhausenia pseudospretella*, Stainton, × 15, to show the strong biting jaws. (1), (2) and (3) after Durrant and Beveridge.

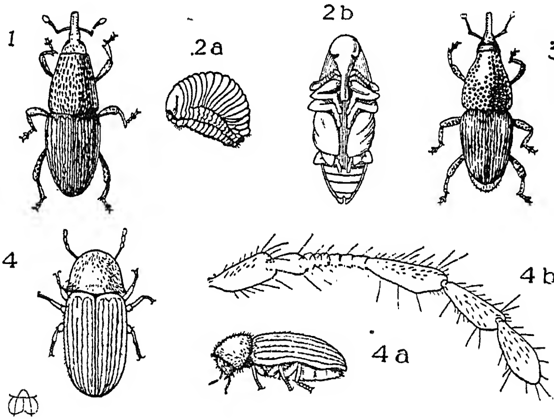


FIG. 15.—Insect Pests found in Flour.

(1) *Calandra granaria*, Linn., × 5. (2*a*) Larva, and (2*b*) Pupa of the same × 5. (3) *Calandra oryzæ*, Fab., × 5. (4) *Sitodrepa panicea*, Thoms., × 5. (4*a*) Side View of the same × 5. (4*b*) Antenna of the same × 50. (1), (2*a*) and (2*b*) after Chittenden.

The drug-room beetle is most readily distinguished from the furniture beetle by the form of its antenna, the second joint of which is twice as long as the third, while in

the furniture beetle these two joints are equal in length (see Fig. 15).

The two weevils are recognized by their long proboscides, to which the elbowed antennæ are attached; *Calandra oryzæ* is distinguished by the four orange-coloured patches (represented by the paler areas in the figure) upon its wing-cases. Leaflet No. 206 published by the Board of Agriculture and Fisheries contains many interesting details about these weevils. The presence of any of these creatures in flour indicates deterioration by long storage.

CHAPTER III

SEDIMENTATION AND CENTRIFUGATION

Sedimentation and centrifugation are processes producing very similar results, and in a general sense one can effect by sedimentation all that can be done by the centrifuge, but a longer time is needed for the operation. The centrifuge, therefore, finds its special use for dealing with liquids, like urine, which putrefy rapidly, or for operations which must be carried out in a short interval of time. Sedimentation is most generally useful for the separation of material suspended in liquids such as water and sewage.

The examination of deposits from urine is fully discussed in such books as Lindley Scott's *Atlas of Urinary Deposits*, or Stitt's *Practical Bacteriology, Blood Work, and Animal Parasitology*, or in the useful little work published by the *Chemist and Druggist*, entitled *Practical Methods of Urine Analysis*.

Collection of Water Deposits. In the case of drinking-waters, half a gallon of the water is allowed to stand until the solids have settled to the bottom, and the clear portion is decanted into a second clean bottle. This should be done as soon as possible after the receipt of the sample of water, because many organisms quickly die and become disintegrated and unrecognizable if the water is kept for any length of time. The sediment and small amount of water remaining are well shaken up and poured out into a conical glass, and allowed to stand for a further period, during which the suspended matter collects in the apex of the cone. A portion of the deposit is removed by means of a cylindrical dipping tube, and placed upon a slide for

microscopical examination. When the deposit is very small in amount or is one that settles slowly, it is better to use a centrifuge, and the form of tube that is most serviceable is that shown in the accompanying figure (Fig. 16). After whirling, the sediment is found in the small detachable tube, and when this is removed the bulk of the clear water remains in the corked upper portion. The deposit is transferred to a slide in the usual way, by means of a dipping tube.

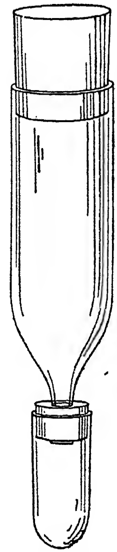


FIG. 16.—Tube for Centrifugation of Water Deposits.

× ½. The capacity of the tube is about 25 c.c.

Dipping Tube. A dipping tube consists of a piece of quill glass tubing about 6 or 8 in. long, having its rough ends smoothed by fusion in the flame. It is most convenient to have several such tubes of different internal diameters, so that one may select a tube suited to the size of the particles to be removed. When in use one end of the tube is closed by the forefinger, while it is supported by the other fingers and the thumb. The open end is dipped into the liquid until it stands over the object one wishes to examine; the forefinger is removed, and the object rises rapidly into the tube, when the finger is replaced on the upper end and the tube is withdrawn from the liquid. It is often a useful plan to hold the tube vertically for a few minutes while the suspended particles settle into the drop which gathers at the end; this is then allowed to fall upon the slide, and a cover-glass is applied. Heavy sandy particles are not always easily brought into the dipping tube by this method, because they sink so rapidly that by the time the tube has been removed from the liquid the sandy particles have fallen from it to the bottom of the vessel. In such circumstances it is a useful plan to adjust the depth of liquid to about ¾ in., then, having

placed the dipping tube over the sandy particles, remove the finger suddenly, quickly replace it, and withdraw the tube rapidly; the sand will not then have time to fall from the tube during the withdrawal, and is retained in the drop at the end of the tube by the surface tension of the liquid.

Examination of the Deposit. The microscopical examination of a water deposit will not yield information of value in every case. Sometimes the deposit is of an indefinite character and very small in quantity, so that no exact conclusion can be deduced from its microscopical appearance. In the majority of instances, however, the deposit is sufficiently characteristic to allow definite inferences to be drawn.

In ordinary circumstances the aim of the microscopical examination of water deposits is to obtain information relative to the origin and environment of a source of water. In most cases the knowledge gained is such as would be learnt by an actual inspection of the surroundings, if it were possible to visit the place from which the water has been taken. From this point of view the microscopical results are most valuable, and frequently afford the key to the interpretation of chemical and bacteriological observations.

It is therefore necessary to study the whole assemblage of plants, animals, and mineral particles noted during the microscopical examination of any specimen of water as a characteristic association, and to consider what are the conditions of which such an assemblage is typical. One must avoid drawing sweeping conclusions from the presence of any one particular organism, and it is not generally necessary to identify exactly the species of each organism present. What one does need is to be able to recognize certain types of deposits as indicative of particular kinds of environment.

Types of Deposit.

N.B.—*The letters and numerals following the names of the organisms refer to the accompanying Plates and drawings.*

The following ten types of deposit may be easily distinguished :—

1. *Rain water.*—Soot and atmospheric dust and the structures produced by the action of moisture and sunshine upon the dust particles. These structures will be such things as filamentous algæ, fungal hyphæ and fern prothalli, which develop from spores carried by the wind on to the roofs of the buildings from which the rain water is collected.

2. *Open spring oozing from grass land.*—Green filamentous algæ (B 8–14, 24), diatoms (A), and ciliated infusorians (C 21–27).

3. *Running water of a brook or river.*—Much sand or clay ; detritus of vegetation ; nematode worms (D 12), diatoms (A).

4. *Moorland water.*—Diatoms (A) ; desmids (B 1–7, 20, 21) ; rotifers (D 7–10) ; sponge spicules ; brown indefinite matter not decolorized by hydrochloric acid ; leaves of sphagnum moss.

5. *Pond water.*—Water-fleas (D 1, 4–6) ; nematode worms (D 12) ; larvæ of aquatic insects, e.g. gnats (E 10) ; and *Chironomus* (E 12) ; green filamentous algæ (B 8–14, 24) ; diatoms (A) ; rhizopods, e.g. *Amœba* (C 13) ; *Arcella* (C 15) ; and *Trinema* (C 16) ; brownish indefinite matter.

6. *Sub-soil water of river alluvium.*—Vorticellæ (C 21) ; Amœbæ (C 13) ; bacteria (C 7–12) ; Paramœcia (C 25) ; gray indefinite matter ; a little sand and clay.

7. *Surface water.*—Water-fleas (D 1, 4–6) ; detritus of vegetation ; nematode worms (D 12) ; diatoms (A).

8. *Contamination by household dust.*—Cotton (Fig. 31), and wool (Fig. 29) fibres, especially coloured ones ; starch grains (Figs. 10, 17, 18) ; barbs of feathers (D 18, 19) ; flakes of skin.

9. *Contamination by sewage.*—White flakes composed of filaments of *Cladothrix dichotoma* (C 5) ; *Beggiatoa* (C 3) ; Bacteria (C 7–12) (such as large bacilli and spirilla, both moving freely and in zoogleal masses) ; Vorticellæ (C 21) ; Paramœcia (C 25) ; Euglenæ (B 32).

10. *Supply carried through an iron pipe.*—*Crenothrix* filaments (C 1), and an amorphous deposit of iron rust.

Examples of Deposits. The following examples of deposits from waters show the kind of information that can be derived from their study.

Deposit 1.—Black amorphous particles (soot); a little sand; green filamentous algæ (B 8–14, 24); germinating fern spores; Vorticellæ (C 21); rotifers (D 7–10); fungal spores; and a small amount of detritus of vegetation.

The soot and other structures present are derived from atmospheric dust washed down by rain, and the deposit is typical of a rain water collected from the roof of a country house.

Deposit 2.—Diatoms (A); ciliated infusorians (C 21–27); *Spirogyra* (B 12); *Gonium* (B 26); *Conferva* (B 8); *Planaria alpina* (E 2).

The absence of sand and the presence of filamentous algæ indicate a still pool produced by a gently flowing spring in a field. Planarian worms are common inhabitants of spring waters.

Deposit 3.—Sand; detritus of vegetation; diatoms; *Paramœcium* (C 25); and *Euplotes* (C 23); rotifers (D 7–10); nematode worms (*Anguillula fluviatilis*) (D 12); monads.

This deposit was obtained from water collected from a small brook.

Deposit 4.—Much sand; numerous diatoms (A); detritus of vegetation; ciliated infusorians (C 21–27); water-fleas (D 1, 4–6); nematode worms (*Anguillula fluviatilis*) (D 12); indefinite matter.

This deposit was from the water of a river.

The two deposits Nos. 3 and 4 are very similar, and are characterized by sand in fairly large amount, detritus of vegetation, and diatoms. Amœbæ, Vorticellæ, and other organisms of stagnant water are absent.

Deposit 5.—Diatoms (*Tabellaria*) (A 13); *Cyclotella* (A 15); *Gomphonema* (A 7); etc.; sponge spicules (C 28);

rotifers (D 7–10) ; desmids (*Tetmemorus* (B 2) ; *Ankistrodesmus* (B 30) ; *Cosmarium* (B 17, 18) ; etc. ; a few grains of sand ; fragments of *Sphagnum ;* brown, indefinite matter.

The water yielding this deposit was from a moor with sphagnum bogs.

Deposit 6.—Sand ; diatoms (A) ; rotifers (D 7–10) ; *Anguillula fluviatilis* (D 12) ; detritus of vegetation ; brown amorphous matter, not decolorized by hydrochloric acid (humus).

The humus colouring matter indicates that the water is derived from a moorland source, and the other features are characteristic of a natural spring running from a hill side.

Deposit 7.—Sand ; nematode worms (D 12) ; detritus of vegetation ; a few diatoms (A) ; rhizopods (*Difflugia protieformis* (C 17–19) ; and *Trinema* (C 16) ; moss protonema ; brown colouring matter of humus.

This deposit is very similar to No. 6, but is modified by the presence of rhizopods, derived from swampy ground, bordering the channel which carries the water from the spring.

Deposit 8.—Numerous planarian worms (*Planaria alpina*) (E 2) ; a little sand ; barb of a feather (D 18, 19) ; fibres of cotton (Fig. 31) ; and wool (Fig. 29) ; some of the woollen ones being coloured red and green ; pinewood tracheids.

Household dust in the form of sawdust, fragments of feathers, and fibres from cloth shows that the water is from a well situated inside a house and badly protected against the admission of sweepings from the floor. The sand and planarian worms indicate that the source is a running spring in a hilly district.

Deposit 9.—Vorticellæ (C 21) ; detritus of vegetation ; large ciliated infusorians (C 21–27).

Deposit 10.—Indefinite matter coloured brown by iron rust ; numerous Paramœcia (C 25) ; and small Amœbæ (C 13) ; numbers of large bacteria.

Deposit 11.—A little sand ; numerous *Coleps* (C 22) ;

(barrel animalcule); zooglea of bacteria (C 10); fungal filaments (C 2, 5); and spores (C 6); nematode worms (D 12); and small Amœbæ (C 13).

The three deposits 9, 10, and 11 are all characteristic of water from wells sunk in river alluvium, and the organisms present are generally colourless, and live in the mud of stagnant water.

Deposit 12.—Fine earth composed of cretaceous foraminifera (see Fig. 27); a little indefinite organic matter and a few infusorians (C 21–27).

From a well sunk in a pure chalk rock.

Deposit 13.—Larvæ of aquatic insects (*Culex*, E 10, and *Chironomus*, E 12); detritus of vegetation; sand; piece of semi-pulped paper; rotifers (D 7–10); Amœbæ (C 13); ciliated infusorians (C 21–27); diatoms (A); indefinite matter.

These structures indicate the free access of surface water and of dust. The organisms present are mainly characteristic of pond life, i.e. stagnant, shallow water. The deposit was from a badly protected, shallow dip well.

Deposit 14.—Chiefly sand, fine earth (clay), and indefinite matter, all coloured brown by iron oxide. Moth scale (D 13–17); dead mite; dead water-flea (*Cyclops*) (D 1); detritus of vegetation; spores of *Septoria*; numerous large bacteria and oscillating filaments.

This deposit resembles No. 13, but does not exhibit the characteristics of a pond. The presence of dead organisms, moth scales, spores of *Septoria* from decayed leaves, etc., shows clearly that it is insufficiently protected from access of dust, and that trees are in the neighbourhood. The water was from a well 72 ft. deep, provided with a windlass and bucket.

Deposit 15.—Diatoms (A); rotifers (D 7–10); Amœbæ (C 13); ciliated infusorians (C 21–27); *Crenothrix* (C 1); filaments and iron rust.

This was from a properly protected well supplied by a

spring carrying some surface water. The *Crenothrix* and iron rust are due to the passage of the water through about thirty yards of iron pipe to the house.

Deposit 16.—Brownish grey indefinite matter; sand; particles of foraminiferous chalk (Fig. 27); detritus of vegetation; diatoms (A); *Gammarus pulex* (E 5); a few starch grains.

The sand and foraminifera show that the water was obtained from a well sunk through a clay soil into a chalky subsoil, while the organisms present indicate the access of surface water.

Starch in Well Waters. The occurrence of starch grains in small numbers is not unusual in waters from wells in farming districts. Most commonly the starch is wheat, barley, or potato, and some of the grains are often eroded. The starch is derived from stray grains of wheat, barley, etc., which have gained an entrance to the well.

Occasionally the starch has a sinister significance; it will then occur in larger quantity, and other indications of a faulty condition will be found, as, for example, a broken trough under the pump, where potatoes are washed in preparation for the kitchen, leading to a leakage into the well of both wash water and starch.

For the characteristics of potato starch, see Fig. 17.

Identification of Organisms. There are cases where the complete identification of organisms is desirable, as in the event of bad odours, choking of pipes, etc., being caused by specific organisms such as *Asterionella* (A 10), and *Crenothrix* (C 1). There are also occasions when growths of *Volvox* (B 15), *Cryptomonas* (B 33), *Microcystis* (B 16) and other algæ have rapidly developed in enormous quantities in reservoirs and created alarm by their presence; it is then important to be able to identify the plant and to give assurance as to its harmlessness or otherwise, and to suggest means by which such troubles may be averted.

The full-page plates inserted at the end of this section

FIG. 17.—Potato Starch.
× 400.

give drawings of a large number of typical plant and animal organisms, by comparison with which it will be possible to form some idea as to the nature of most of the microscopic structures present in water deposits. The particular species illustrated in the figures are those which occur frequently in drinking water supplies and will enable the worker, in most instances, to arrive at a fairly correct conclusion as to the group or even the genus to which the organisms he is examining belong.

For further information and for the precise identification of organisms the worker should consult monographs dealing with special groups, such as *Algæ*, Vol. 1, by G. S. West; *British Freshwater Rhizopoda and Heliozoa*, Vols. 1 to 5 (Ray Society), by Cash and Wailes; *The British Desmidaceæ*, Vols. 1 to 4 (Ray Society), by W. West and G. S. West; and *The Rotifera*, Vols. 1 and 2, by Hudson and Gosse. See also the books named under the heading "Water Deposits" in the general bibliography at the end of this book.

The drawings on the following five plates have been made with as small a number of magnifications as possible. The magnifications used are 2½, 20, 100, 200 and 400 diameters and organisms of the same type are drawn to the same magnification so as to give an idea of relative size. In figuring the algæ, it was necessary to use three magnifications, viz., 400 for diatoms and 100 and 200 for other algæ; the drawings at particular magnifications are, however, grouped together.

PLATE A.

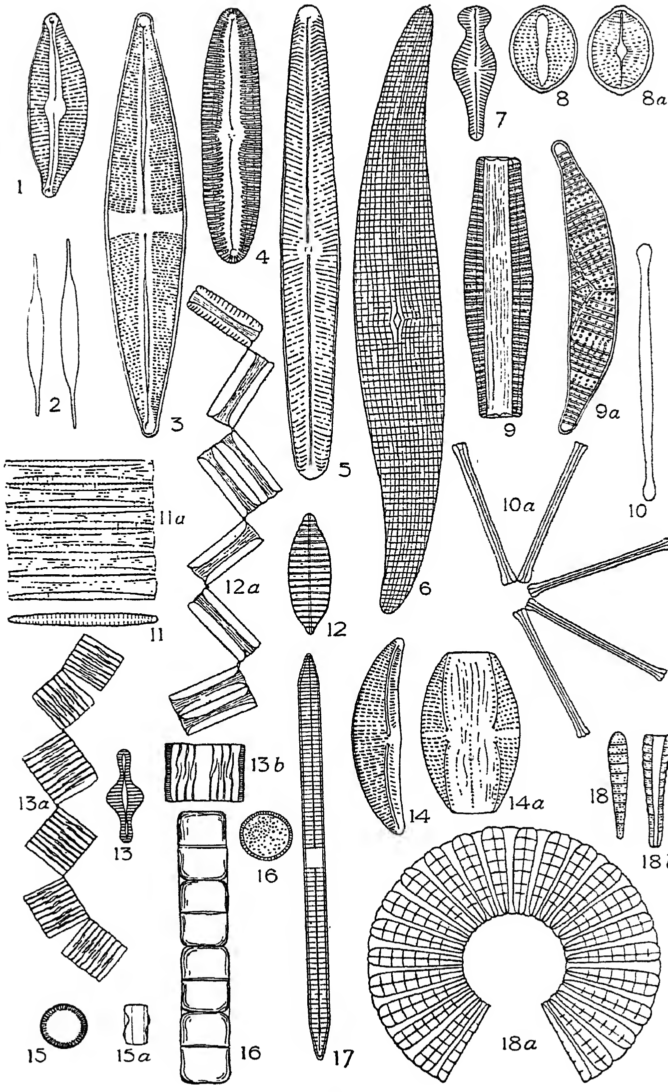

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PLATE A.

Diatoms commonly found in Drinking Waters. All × 400. After H. van Heurck.

1. Cymbella Ehrenbergii, Kütz., valve view.
2. Nitzschia acicularis, Wm. Sm., valve view.
3. Stauroneis Phœnicenteron, Ehr., valve view.
4. Navicula viridis, Kütz., valve view.
5. Navicula oblonga, Kütz., valve view.
6. Pleurosigma attenuatum, Wm. Sm., valve view.
7. Gomphonema constrictum, Ehr., valve view.
8. Cocconeis Pediculus, Ehr., valve view.
8*a*. „ „ „ girdle view.
9. Epithemia turgida, (Ehr.) Kütz., valve view.
9*a*. „ „ „ „ girdle view.
10. Asterionella formosa, Hassall, var. gracillina, valve view.
10*a*. „ „ „ „ girdle view.
11. Fragilaria capucina, Desmazières, valve view.
11*a*. „ „ „ girdle view.
12. Diatoma vulgare, Bory, valve view.
12*a*. „ „ „ girdle view.
13. Tabellaria flocculosa, (Roth.), Kütz., valve view.
13*a*. „ „ „ „ girdle view.
13*b*. „ „ „ „ „ „
14. Amphora ovalis, Kütz., valve view.
14*a*. „ „ „ girdle view.
15. Cyclotella Kützingiana, Chauvin, valve view.
15*a*. „ „ „ girdle view.
16. Melosira varians, Ag., valve view.
16*a*. „ „ „ girdle view.
17. Synedra Ulna (Nitzsch.), Ehr., valve view.
18. Meridion circulare, Ag., valve view.
18*a*. „ „ „ girdle view.
18*b*. „ „ „ girdle view.

PLATE B.

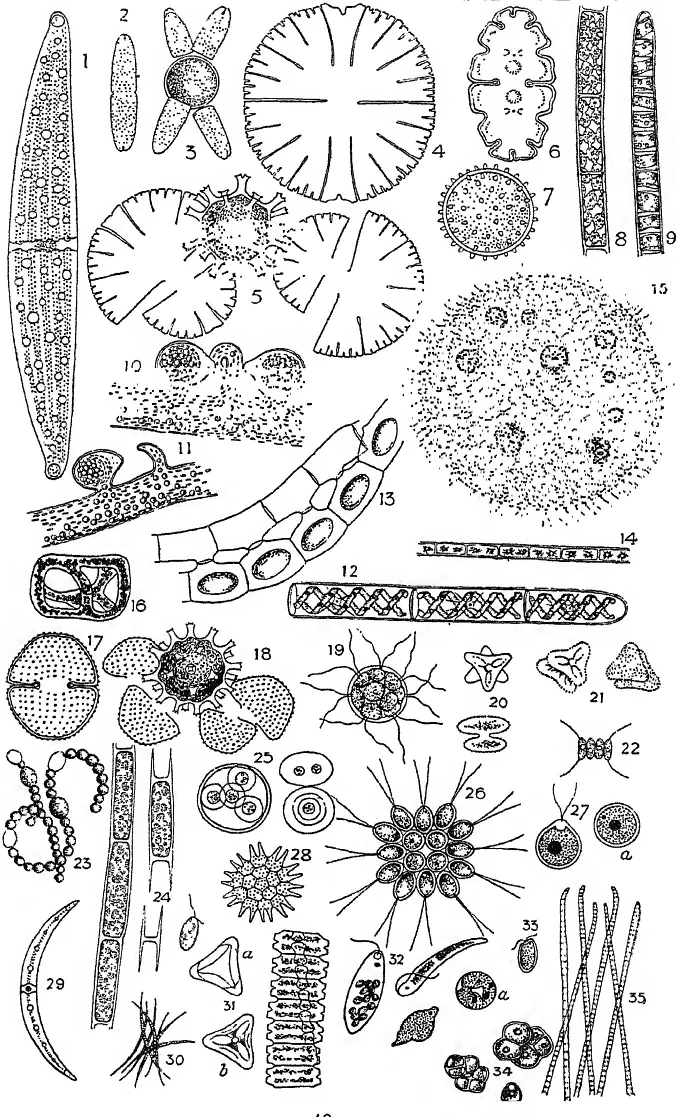

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PLATE B.

Algæ and Flagellates found in Drinking Waters.

1 to 16, × 100; 17 to 35, × 200.

1.	Closterium lunula, Ehr.	Chlorophyceæ, Akontæ, Desmidaceæ
2.	Tetmemorus granularus (Breb.), Ralfs.	,, ,, ,,
3.	,, ,, ,, ,, with zygospore	,, ,, ,,
4.	Micrasterias denticulata, Breb.	,, ,, ,,
5.	,, ,, ,, with zygospore	,, ,, ,,
6.	Euastrum oblongum (Grev.), Ralfs.	,, ,, ,,
7.	,, ,, ,, ,, with zygospore	,, ,, ,,
8.	Rhizoclonium hieroglyphicum, Kütz., formerly known as Conferva	,, Isokontæ.
9.	Ulothrix zonata (Web. & Mohr.), Kütz.	,, ,,
10.	Vaucheria sessilis (Vauch.) D.C.	,, ,,
11.	,, ,, ,, ,,	,, ,,
12.	Spirogyra porticalis, Vauch.	,, Akontæ.
13.	,, ,, ,, in conjugation	,, ,,
14.	Zygnema stellinum (Vauch.) Ag.	,, ,,
15.	Volvox aureus (Vauch) D.C.	,, Isokontæ.
16.	Microcystis (Clathrocystis) æruginosa, Henfrey	Cyanophyceæ, Coccogoneæ.
17.	Cosmarium Botrytis, Menegh.	Chlorophyceæ, Akonteæ, Desmidaceæ
18.	,, ,, ,, with zygospore.	,, ,, ,,
19.	Pandorina Morum (Mull.) Bory	,, Isokontæ.
20.	Stauastrum dispar.	,, Akontæ, Desmidaceæ.
21.	Stauastrum punctulatum, Breb.	,, ,, ,,
22.	Scenedesmus quadricauda (Turp.), Breb.	,, Isokontæ.
23.	Anabœna Flos-aquæ, Brebisson	Cyanophyceæ, Hormogoneæ.
24.	Tribonema bombycina (Ag.) Derb. & Sol., formerly known as Conferva.	Chlorophyceæ, Heterokontæ.
25.	Gleocystic gigas (Kütz.) Lagerh.	,, Isokontæ.
26.	Gonium pectorale, Mull.	,, ,,
27.	Chlamydomonas sp. (*a*) resting stage.	,, ,,
28.	Pediastrum Boryamum (Turp.) Menegh.	,, ,,
29.	Closterium parvalum.	,, Akontæ, Desmidaceæ.
30.	Ankistrodesmus falcatus (Corda) Ralfs.	,, Isokontæ.
31.	Desmidium Swartzii, Ag. (*a*) and (*b*), end views of the same.	,, Akontæ, Desmidaceæ.
32.	Euglena viridis, Ehrenberg, (*a*) resting stage.	Flagellata.
33.	Cryptomonas sp.	,,
34.	Pleurococcus vulgaris, Menegh. or Protococcus viridis, Ag.	Chlorophyceæ, Isokontæ.
35.	Oscillatoria tenuis, Ag.	Cyanophyceæ, Hormogoneæ.

PLATE C.

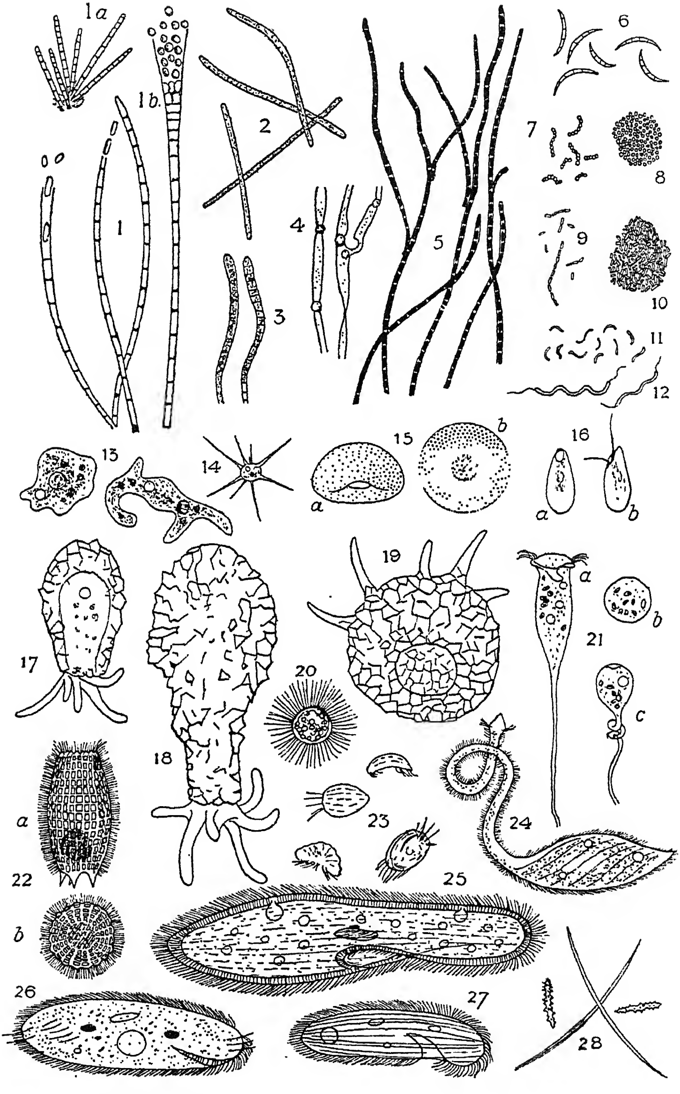
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PLATE C.

Fungi and Protozoa. All × 200.

1.	Crenothrix polyspora, Cohn.	Schizomycetes.
1*a*.	Colony of young Crenothrix threads.	,,
1*b*.	Trumpet-shaped sheath, with spores.	,,
2.	Leptohtrix ochracea, Kützing.	,,
3.	Beggiatoa alba, Vaucher.	,,
4.	Leptomitus sp.	,,
5.	Cladothrix dichotoma, Cohn.	,,
6.	Fusarium sp., spores.	,,
7.	Streptococcus.	Bacteria.
8.	Micrococcus.	,,
9.	Bacillus.	,,
10.	Zooglœa of Bacilli.	,,
11.	Vibrios.	,,
12.	Spirilla.	,,
13.	Amœba proteus, Leidy. *a* and *b*, two positions of the same organism.	Amœboid Protozoa.
14.	Dactylosphærium radiosum (Ehr.) Bütschli.	,, ,,
15.	Arcella vulgaris, Ehrenberg. *a*, side view; *b*, under surface.	,, ,,
16.	Trinema enchelys, Ehr. *a*, front view; *b*, side view.	,, ,,
17.	Difflugia Penardi, Hopk.	,, ,,
18.	Difflugia oblonga, Ehrenberg.	,, ,,
19.	Centropyxis aculeata, Stein.	,, ,,
20.	Actinophrys sol, Ehrenberg.	,, ,,
21.	Vorticella nebulifera. *a*, extended; *b*, contracted, seen from above; *c*, contracted, seen from the side.	Ciliate Protozoa (Infus
22.	Coleps hirtus, Ehrenberg (Barrel animalcule). *a*, side view; *b*, end view.	,, ,, ,,
23.	Euplotes charon, Müller. Four positions.	,, ,, ,,
24.	Lacrymaria olor, Müller (Swan animalcule).	,, ,, ,,
25.	Paramœcium aurelia, E. (Slipper animalcule).	,, ,, ,,
26.	Pleurotrica lanceolata, Ehr.	,, ,, ,,
27.	Colpidium striatum, Stokes.	,, ,, ,,
28.	Spongilla lacustris, Linn. Spicules.	Porifera (Sponges).

PLATE D.

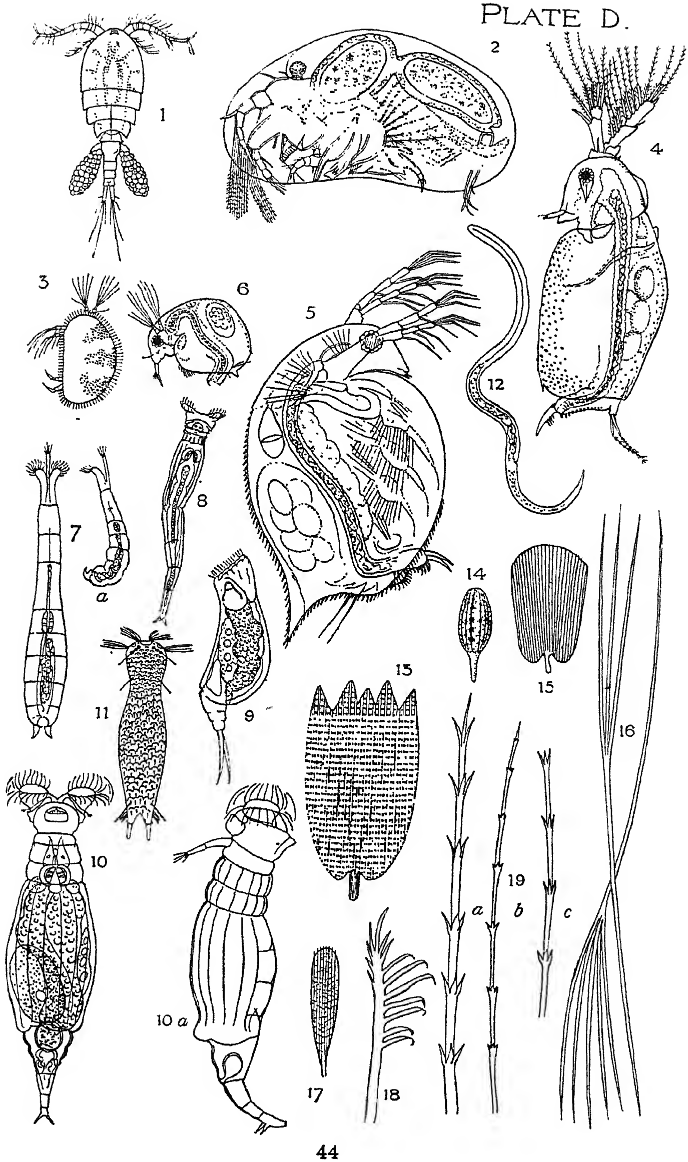

PLATE D.

Water-fleas, Rotifers, Moth scales, etc. 1 to 6, × 20; 7 to 15 and 17 to 19, × 200; 16, × 100.

1.	Cyclops vulgaris.	Copepoda (Water Flea).
2.	Cypris virens, Jurine.	Ostracoda.
3.	Cypridopsis vidua, O. F. Müller.	,,
4.	Sida crystallina, O. F. Müller.	Cladocera (Water Fleas).
5.	Daphnia pulex, de Geer.	,, ,,
6.	Bosmina longirostris, O. F. Müller.	,, ,,
7.	Rotifer macroceros.	Rotifera (Wheel Animalcules).
8.	Rotifer vulgaris, Schrank.	,, ,, ,,
9.	Proales tigridia.	,, ,, ,,
10.	Philodina acuticornis, Murray. Dorsal view.	,, ,, ,,
10*a*.	Philodina acuticornis, Murray. Lateral view.	,, ,, ,,
11.	Chætonotus brevispinosus, Zelinka.	Gastrotricha.
12.	Nematode worm, commonly known as Anguillula fluviatilis.	
13.	Scale of Papilio machaon (Sulphur yellow butterfly).	
14.	Scale of Polyommatus argiolus (Azure blue butterfly), battledore scale.	
15.	Scale of Polyommatus argiolus, ordinary scale.	
16.	Hair-like scales of Tinea pellionella, the Clothes Moth.	
17.	Scale of Culex pipiens, the Common Gnat.	
18.	Hooked pinna of a Goose feather.	
19*a*, *b*, and *c*.	Barbs of down feathers.	

PLATE E.

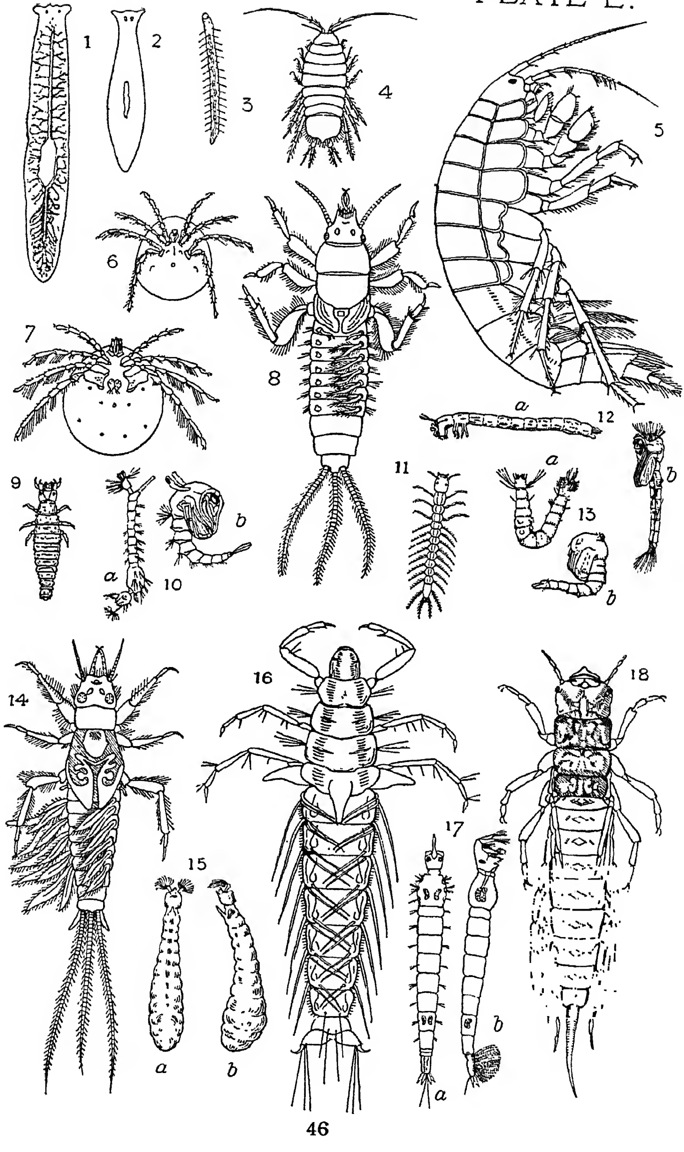

PLATE E.

Worms, Mites and Insects. All × $2\frac{1}{2}$.

1.	Dendrocoleum lacteum, Oersted.	Turbellaria, Vermes.
2.	Planaria alpina.	,, ,,
3.	Nais communis, Piguet.	Chætopoda, Vermes.
4.	Asellus aquaticus.	Malacostraca, Crustacea.
5.	Gammarus pulex.	,, ,,
6.	Eylais (Eulais) extendens, Müller.	Hydracarina (Water Mites).
7.	Hydrachna geographica, Müller.	,, ,, ,,
8.	Ephemera vulgata, Linn. (May fly), Larva.	Insecta.
9.	Hydrobius fuscipes, Larva.	,,
10.	Culex pipiens (the common gnat). *a*, larva ; *b*, pupa.	,,
11.	Gyrinus natator (whirligig beetle), larva.	,,
12.	Chironomous dorsalis (harlequin fly). *a*, larva ; *b*, pupa.	,,
13.	Dixa sp. *a*, larva ; *b*, pupa.	,,
14.	Polymitarcys virgo (a May-fly). Larva.	,,
15.	Simulium reptans. Larva, *a*, front view ; *b*, side view.	,,
16.	Phryganea varia (the Caddis fly), larva.	,,
17.	Corethra plumicornis (Phantom larva). *a*, dorsal view ; *b*, side view.	,,
18.	Sialis lutarius, L. Larva.	,,

Sedimentation in Liquids Other than Water. In certain cases it is advantageous to use liquids of a greater or less density than water for effecting a concentration of constituents by sedimentation. For example, if one prepares a solution of common salt by dissolving 23 gms. of salt in 77 c.c. of water, it will be found that when a little is shaken in a tube with some ground roast coffee the liquid remains colourless, while the coffee floats almost entirely to the top. When ground roast chicory is similarly treated the liquid turns yellowish-brown, and the greater part of the chicory sinks. If, then, a coffee contains a smaller amount of chicory than is easily recognizable by making a simple microscopical mount, it can be rapidly concentrated by shaking the powdered coffee with the salt solution, allowing to stand for 15 to 30 minutes, and removing the floating layer by carefully filling up the tube with salt solution till it overflows and carries away all the coffee. The residue at the bottom of the tube will be found to consist almost wholly of chicory, which can be removed and examined microscopically. A mixture of two volumes of glycerin with one volume of water produces a liquid having a similar density to that of the salt solution described above. This diluted glycerin answers equally well for separating chicory from coffee, and will often be preferred because it is rather easier to prepare, and it leaves no solid residue on evaporation.

The details of the structural elements by which coffee and chicory may be distinguished are described and figured on pp. 67–69.

A similar method of procedure has been advocated by C. G. Hinrichs in a pamphlet entitled *Method for the Proximate Analysis of Powders, Especially Baking Powders.* December, 1901. He separates a mixture like a baking powder by shaking it with a chloroform-bromoform mixture, of density 1·8, and centrifuging. All the starch rises as a cream, and is skimmed off, filtered, washed with chloroform, dried, and kept for microscopic examination.

The residue which settled is now stirred up with other mixtures of chloroform and bromoform, gradually increasing in density, and the various floating layers separated and reserved for further examination. By this means it is possible to separate the various constituents in a pure condition and to say definitely in what combinations the different acids and bases were present.

The use of the same process has been extended by Netolitzky (*Biochem. Zeitsch.*, 1919, 93, 226–230) to the separation of constituents of powdered drugs. For example quillaia bark contains a large amount of crystals of calcium oxalate, CaC_2O_4,H_2O, which has a density of 1·83. These crystals will, therefore, sink in chloroform (density 1·5), while the less dense cellulose and cell-contents will float. Hence, by shaking finely powdered quillaia bark with chloroform, and centrifuging or allowing to settle, a deposit consisting of almost pure calcium oxalate in the form of crystals is obtained, and can be further purified by shaking with chloroform, carbon tetrachloride, or bromoform, or mixtures of bromoform with alcohol or ether. Netolitzky states that crystals from sesame seeds can be similarly separated, as also aleurone grains and other similar substances, from various powdered plant products.

CHAPTER IV

ELUTRIATION AND THE USE OF SOLVENTS

Application of Elutriation. Returning now to the discussion of methods of concentrating material for microscopic examination, the process of elutriation claims attention. This process may be regarded as a combination of partial sedimentation with decantation, by which means finer particles are separated from coarser or specifically lighter ones from those which are more dense.

A good example of its application is found in the method of examining rice for facing. This is done by vigorously shaking a few grammes of the rice with a small volume of water, pouring off the turbid liquid, allowing this to settle, and removing a small portion of the sediment for microscopical examination. If the rice has been "faced," particles of talc will be found in the sediment, which should be compared with a standard preparation made by mounting an authentic specimen of talc in water. If the turbid liquid contains much starch, the talc present may be concentrated by digestion with diluted hydrochloric acid, after which the mineral residue, if any, can be examined microscopically.

Elutriation with water is also used to establish the foraminiferous nature of a chalky limestone. The limestone is gently rubbed under water with a camel-hair brush; the turbid liquid is stirred up and allowed to settle partially. The smaller suspended particles are poured away with the supernatant liquid, and the foraminiferous shells are found in the residue. These shells have forms

similar to those shown in Fig. 27, p. 81. A satisfactory mount can be made by drying a portion of the sediment in a tube in the steam oven, adding some turpentine to the dry residue, and boiling to expel air. If a drop is now transferred to a slide and covered with a cover-glass the structure of the shells is clearly seen. To make a permanent preparation, add a drop of xylol-balsam before applying the cover-glass.

Sago and Tapioca. Sago and tapioca are most readily examined as to the nature of their starch by a similar method. The material is shaken vigorously in a test-tube partly filled with distilled water and the turbid liquid is poured off. After allowing time for settling, the starch is removed by means of a dipping tube, and mounted in dilute glycerine for microscopic identification.

A variety of pearl tapioca is not infrequently sold as sago and an examination carried out as described would quickly detect any such substitution with absolute certainty. The characteristic features of these two starches are shown in Fig. 18.

Both starches consist of grains, the majority of which were originally compound, but have become largely broken into single grains during the process of manufacture of the commercial article. Sago starch grains are much larger, varying in size from 20 to 55 microns with a maximum of about 65 microns, while tapioca starch grains vary from 15 to 25 microns, reaching a maximum of about 35 microns. Both starches show many muller-shaped grains, but sago starch grains are more ovate in shape, while tapioca starch grains more usually show a circular outline.

Treatment with Solvents. Another group of methods of treatment results in the removal from the material to be examined of certain constituents by the action of solvents. These operations are best carried out by treating comparatively large amounts of the substances in test-tubes, beakers, flasks, or other suitable apparatus. The residue,

which is generally only a small proportion of the original material, is examined as to its microscopic structure.

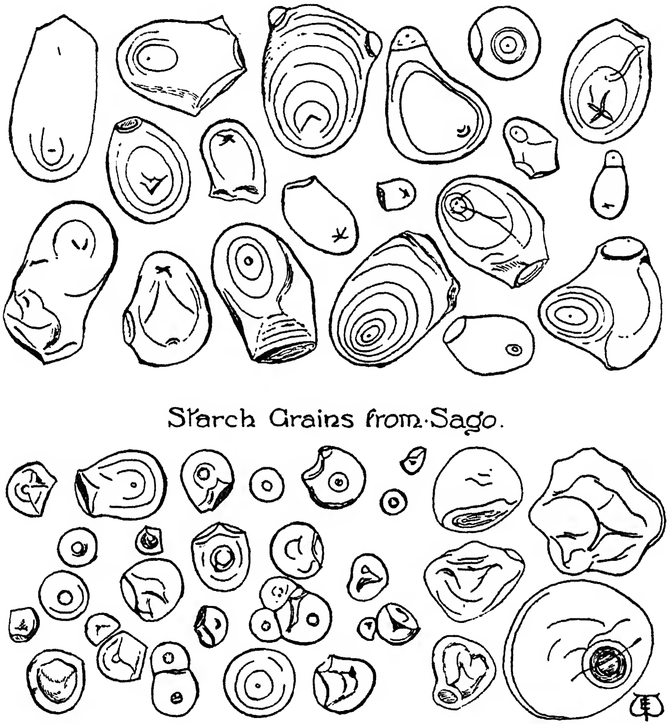

FIG. 18.—Starch Grains from Sago (*Metroxylon Sagu*, Rottb.) and Tapioca (*Manihot utilissima*, Pohl).

The large grains on the right of the tapioca starch have been partially gelatinized by the heat applied in the process of preparation. All × 400.

For the preliminary treatment of sugars, jams, honey, many lozenges and tablets and similar things, water is a useful and sufficient solvent.

Sugars, Jams, and Honey. The insoluble matter from sugar of various kinds will almost always contain a few sugar mites, and in addition one may find fragments of wood, vegetable tissues, and, in dirty or carelessly handled and ill-stored samples, there will also occur such adventitious matters as starch grains, fungal spores, flakes of skin, and similar objectionable matters.

Jams yield a deposit consisting of the more resistant parts of the fruits from which they have been prepared. These parts are the epidermis, which sometimes has characteristic hairs, as in the raspberry, the stones and portions

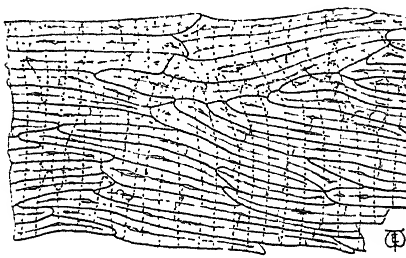

FIG. 19.—Endocarp of Apple.
× 200.

of the calyx and other flower parts, such as the style in strawberries and raspberries. Owing to the vigorous boiling to which the fruits are subjected during the preparation of jams, all these structures are in an excellent condition for microscopic examination, and can be compared with material obtained from authentic specimens of the various fruits and with drawings such as are to be found in Winton's *Microscopy of Vegetable Foods*. Apple pulp added to jam can often be identified by searching for pieces of the tough membrane (endocarp) from the core, which has a very characteristic appearance under the microscope. This endocarp (see Fig. 19) consists of elongated and lignified sclerenchymatous cells arranged in groups meeting at

various angles, and in two or more layers, with their long axes more or less at right angles to one another. The cells vary in length from 80μ to 210μ, and in breadth from 5μ to 12μ, the lumen being represented by little more than a line. Occasionally one finds a cell with a much larger lumen and wider in proportion to its length. Another method for the detection of apple pulp in jams will be considered later (see p. 80).

Rhubarb in jam is recognized by the presence of large wood vessels with spiral, annular, and reticulate thickening, and cluster crystals of calcium oxalate, the diameter of which reaches 90μ. There will also be found occasional prismatic crystals, some rather loosely formed clusters, and a few minute linear prisms. In some of the older books (vide *Micrographic Dictionary*) it is stated that rhubarb petiole contains acicular raphides ; but such is not the case. This error is noted by Solereder (*Anatomy of Dicotyledons*, II., p. 671), who states that large cluster crystals only are present. This is not quite correct, for, although most of the crystals are in clusters, one finds occasionally prismatic crystals, sometimes very well formed (see Fig. 20).

Genuine honey, when dissolved in water, gives a residue consisting almost entirely of pollen grains, so that extraneous matters of the nature of dirt—e.g. pieces of wood and fibres—are easily recognized.

Lozenges, Tablets, and Pills. Lozenges and tablets, when soaked in water, become disintegrated, and yield their sugar and other soluble constituents to the solvent, while vegetable powders and insoluble ingredients form a sediment suitable for immediate examination, and for further treatment with other reagents, such as acids, chloral hydrate, acid and alkali, in succession, etc., as may seem desirable. To clear such a sediment with chloral hydrate some of the thick deposit is removed to a second clean test-tube by means of a dipping tube, and boiled with two or three times its volume of chloral hydrate

solution. The deposit from the chloral is obtained either by sedimentation or centrifugation, and is examined in the usual way.

Pills should first be stripped of their coating by carefully paring it off with a sharp knife, and the remainder is

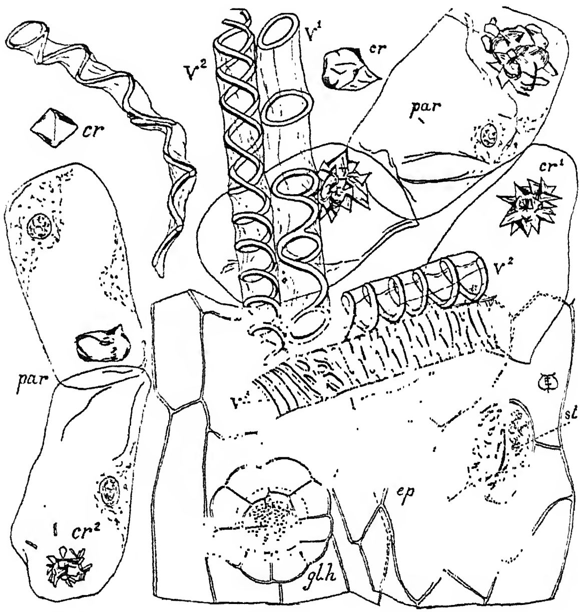

FIG. 20.—Structures from the Petiole of Rhubarb (*Rheum Rhaponticum*).

par, large-celled parenchyma, containing crystals—v^1, annular; v^2, spiral; v^3, reticulate vessels; *cr.*, prismatic; cr^1, cluster; cr^2, loose cluster, with some small prismatic crystals of calcium oxalate; *ep*, epidermis; *st*, stoma; *gl h*, glandular hair. All × 200.

dropped into water in a test-tube, and soaked until disintegrated. The sediment is examined in water, and a part is removed to another tube, and boiled with chloral hydrate or treated with other clearing reagents, after which the new sediment is removed for examination. If

information is desired as to the nature of the coating, the parings should be soaked in water in a separate tube, and the deposit examined in the same way as the pill mass itself. Starch and talc are the most usual ingredients detectable by the microscope. Many trade pills are coated with talc only. Lycopodium spores (see Fig. 44, p. 128) and cinnamon powder are also occasionally used for dusting pills.

CHAPTER V

SOLVENTS AND CLEARING REAGENTS

Parasites of Mange. A weak solution of caustic soda is another very useful reagent for the preliminary treatment of certain specimens. For example, in examining horse-hair, sheep's wool, and other similar materials for mange parasites, it is a good plan to place a quantity of the hair and scrapings in a small beaker-flask with some 2 per cent. caustic soda solution and heat for an hour or so on a water-bath, the beaker being covered by a watch-glass and supported by its lip, so that it is entirely immersed in the steam. This treatment results in the disintegration and solution of the hair, skin, and coagulated blood serum, while the highly resistant chitinous skin of the mites is unattacked. Pour the resulting liquid into a test-tube or other suitable vessel and allow to settle; examine the sediment for mites. If one wishes to hasten the process of sedimentation a centrifuge may be used. The mites are thus concentrated into a small bulk, and the risk of missing them when few in number is greatly diminished. The mites of the three principal types of mange, viz., the symbiotic, psoroptic, and sarcoptic forms, are illustrated in the accompanying figure (Fig. 21). The most virulent form of mange is the sarcoptic; next comes the psoroptic form, while the least harmful is the symbiotic variety. The mites are most readily distinguished by an examination of the suckers or ambulacra, as they are called, with which the legs are furnished; enlarged drawings of these are given.

Canned Fish and Fish Pastes. For the identification

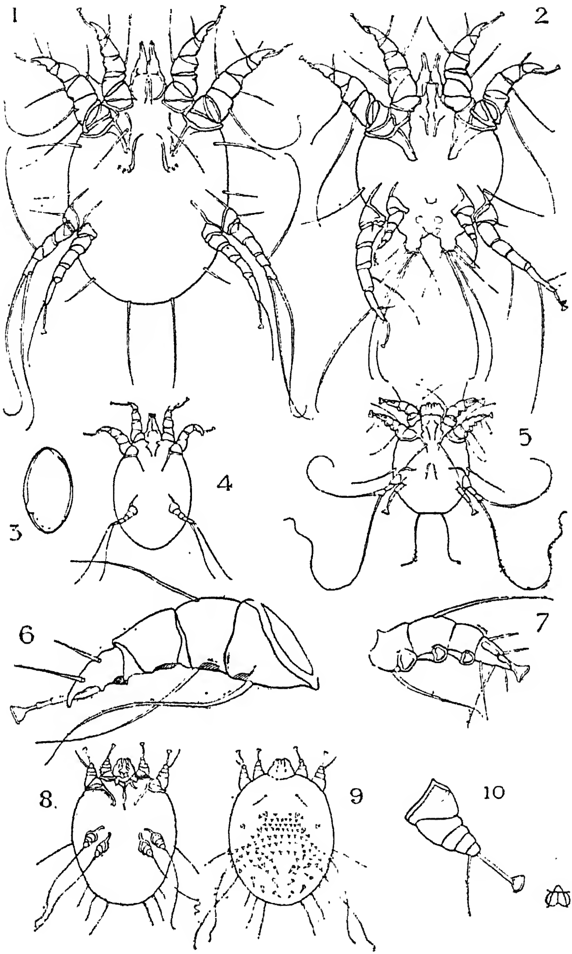

FIG. 21.—Mange Mites.

(1) Female. (2) Male of *Psoroptes equi*, Gervais. (3) Egg and (4) larva of the same. (5) *Symbiotes equi*, Gerlach, female. All × 40. (6) Second leg of female *Psoroptes* × 100. (7) Second leg of *Symbiotes* × 100. (8 and 9) Ventral and dorsal surfaces respectively of female *Sarcoptes equi*, Gerlach, both × 40. (10) Second leg of the same × 100.

of the fish sold in tins, one uses the characters of the scales, an examination of which frequently yields valuable results, even when it is impossible to form a definite opinion as to the actual species present. Some of the scales, which are often very brittle, are carefully removed from the canned fish, cleared by cautiously warming them with 5 per cent. caustic soda solution, washing thoroughly with water and mounting in water or in dilute glycerine.

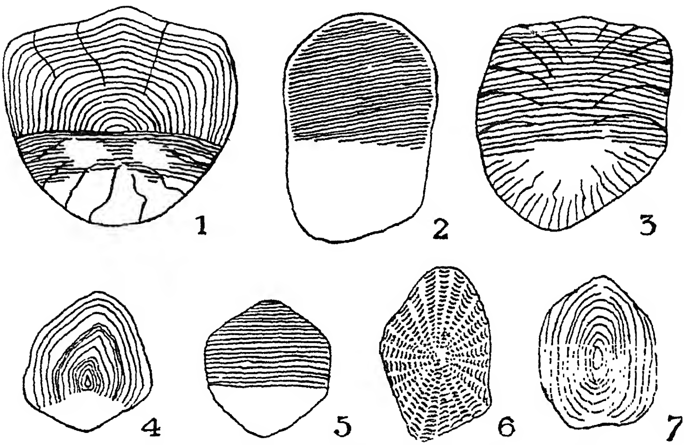

FIG. 22.—Scales of Fish used for Tinning and Canning.

(1) Anchovy, *Engraulis encrasicholus*. (2) Herring, *Clupea harengus*. (3) Pilchard (Sardine), *Clupea pilchardus*. (4) Salmon, *Salmo salar*. (5) Sprat (Bristling) *Clupea sprattus*. (6) Cod, *Gadus morrhua*. (7) Hake, *Merluccius vulgaris*. All × 3, after Essery.

The scales of the fish that are commonly used for canning show wide differences in form and in detailed structure, as will be evident from the accompanying figures. It is wise to mount authenticated scales in glycerine jelly or in Canada Balsam and to keep them for reference; when this has been impossible, the drawings would prove helpful.

Fish pastes are more difficult to handle and the following method of treatment is recommended by Essery in the *Analyst* for April, 1922 :—

"The paste (about 10 grms.) may be mixed to a cream with aqueous 5 per cent. sodium hydroxide solution and diluted to about 150 c.c., and the mixture gently warmed on a steam bath, with frequent stirring. Under these conditions fragments of the scales and bones sink to the bottom, while the rest of the material becomes flocculent, and can be decanted. The scale fragments are then washed thoroughly by decantation, and examined in water. Although, as a rule, reserve is necessary in reporting, in some cases the mere presence of bone and scale fragments is sufficient to indicate adulteration, as for instance their presence in lobster and shrimp pastes, where (these animals being crustaceans) their detection is clear evidence of admixture. A 2-in. power is usually sufficient, any higher power being seldom required."

Use of Solvents for Fats and Oils. Solvents such as chloroform, xylol, petroleum ether, and carbon bisulphide enable one to remove fats from ointments and similar preparations, and also from oily substances such as mustard, pepper, cocoa, and linseed. For this purpose extraction in a Soxhlet's extractor is the most thorough method, but for powders like pepper and mustard one may shake the powder with the solvent, pour it upon a filter-paper supported in a funnel, allowing it to drain, and washing away the remaining fat by pouring on fresh quantities of the solvent. By these means a concentration of the medicament is effected in the case of ointments, and a similar effect is produced in a less degree upon the oily powders, which are also rendered more easy of attack by aqueous liquids during any subsequent treatment to which they may need to be subjected.

Clearing Reagents. Another group of substances produces results very similar to that of actual concentration. These are certain clearing reagents, the most useful of which are chloral hydrate and phenolic substances.

Chloral Hydrate. The chloral hydrate solution is made by dissolving 5 parts by weight of chloral hydrate in

2 parts by volume of distilled water. The use of this solution is indicated when one wishes to bring about the expansion of collapsed cells and to dissolve certain cell contents, such as starch and proteid matter. When starch is present the material should be boiled with the chloral hydrate. Any space left under the cover-glass as a result of the boiling should be filled up by running in some dilute glycerin. This reagent is therefore very useful to make evident the structure of delicate tissues which, during the drying of vegetable substances, tend to shrink until the cell-lumina disappear. Chloral hydrate solution is generally preferable to caustic soda or potash, because it produces less swelling of cellulose walls and is less destructive in its action upon certain cell contents, such as calcium oxalate. For all kinds of vegetable substances—whether fresh or dried or preserved in alcohol—chloral hydrate is the most generally useful clearing reagent; it clears and expands tissues without producing marked distortion, and it is very useful for exhibiting small crystals such as are found in the parenchyma of gentian root.

Drug Extracts. Another instance of the use of chloral hydrate is for the identification and examination of extracts made from drugs. The details of the process are thus given by Greenish and Griffiths (*Pharm. Journ.*, December, 1908, p. 834) :—" 0·2 gm. of the extract was triturated in a mortar with 5 c.c. of water; the turbid liquid produced was transferred to a conical centrifuge tube and centrifuged for about two minutes. The supernatant liquid which was still turbid was poured off, and the deposit stirred up —if necessary, with water—and again centrifuged. It was then mixed with chloral hydrate solution and once more centrifuged. The final deposit was mixed with one or two drops of chloral hydrate solution or glycerin, and small quantities transferred by means of a small pipette to a slide for microscopical examination." Fragments of the tissues of the drugs from which the extracts have been prepared are found in the material on the slides, and in this way the

identity of the extract and its freedom from sophistication can be established.

Phenols. Among phenols, cresol and liquefied phenol behave very similarly; they produce less swelling than chloral hydrate, and are without destructive action upon starch, which, however, is readily penetrated by these phenols, and is rendered so transparent as to be practically invisible, while the tissues themselves are clearly defined. Examined by polarized light, the starch grains in such preparations show a well-marked cross, and crystals are thrown into prominence. Liquefied phenol is useful for a preliminary survey of any non-oily powder, such as self-raising flour, ginger, and many drugs, and is preferable to clove oil in that it is more easily cleaned off the slides by means of water.

Cresol, either alone or mixed with half its volume of glycerin, is a very useful reagent. Vegetable powders are well cleared by its use, and it is markedly successful for showing the structure of denser structures such as the bases of the hairs from the seed coat of nux vomica, the dense masses of sandy crystals in belladonna leaves and root, and the large cluster crystals found in stramonium and rhubarb. Cresol is also very suitable for pollens.

Mounting Insect Parts. It is occasionally necessary to prepare and mount parts of insects. For this purpose the structures are subjected to the clearing action of a succession of clearing and dehydrating agents and are finally mounted in Canada balsam or sandarac.

The specimens are first soaked in 10 per cent. caustic soda until they are thoroughly relaxed, after which they are washed free from alkali by soaking in several changes of water. They are then transferred to glacial acetic acid and, if they are to be mounted in Canada balsam, they are passed to phenol liquefied with alcohol and then to benzol—or xylol-balsam. If they are to be mounted in sandarac, transfer them from the glacial acetic acid in succession to amylic alcohol, phenol liquefied with amylic

alcohol and mount in amyl sandarac (see p. 74) by the exposure method. This is accomplished as follows: A cover-glass is supported on a cork whose diameter is 4 to 6 mm. less than that of the cover-glass and the insect parts are taken from the amyl-phenol and arranged in position; sufficient amyl-sandarac is added to cover the object and the preparation is put aside, in a place free from dust, until most of the solvent has evaporated. Further quantities of mountant are added until the object is embedded in a layer of resin. A drop of amyl-sandarac is placed in the centre of a clean slide and a second drop on the resin covering the object; the cover-glass is carefully picked up and inverted upon the slide. If the mountant shrinks away under the cover, it is replaced by fresh quantities allowed to flow underneath by capillary attraction.

If the insect parts have an appreciable thickness, it is a good plan to select three clear glass beads about equal in thickness to the object to be mounted. These are attached to the cover-glass near its edge, at equidistant points, by means of small drops of the mountant. After allowing them to become fairly dry, one proceeds with the mounting in the usual way.

Oily Powders and Ointments. Clove oil is particularly useful for the preliminary examination of oily powders like pepper, mustard, linseed, etc., because the oily matter dissolves completely and the globules do not obscure the other structures present. It causes no swelling, but has rather a tendency to harden and shrink tissues. It has a great penetrating power, and gives very good effects with polarized light. Clove oil also shows no tendency to crystallize as is the case with chloral hydrate and liquefied phenol, especially when it has been necessary to warm the preparations to gelatinize starch or to thoroughly expel the air. The oil is best cleaned from the slides by wiping both the cover-glass and the slide with paper and polishing with a duster.

Ointments can be readily subjected to a preliminary examination by placing a small amount on a slide and adding a drop or two of clove oil; a cover-glass is applied and the preparation is gently warmed. Any vegetable constituents, such as powdered galls or starch, are rendered transparent and easy of identification, while the fatty constituents become practically invisible.

Crystalline Matter in Flour and Other Materials. Crystals naturally present in starchy materials or powdered crystalline chemicals added to starchy substances, such as is done in the preparation of self-raising flours, are sometimes difficult to detect by ordinary methods, partly because of their small amount and partly because they may be soluble in various aqueous clearing reagents. Phenolic substances have generally no solvent action upon these crystalline materials, while they render starch and proteid almost invisible. For these reasons a self-raising flour is most quickly distinguished from ordinary flour by examining a small quantity mounted in cresol or clove oil, when the added crystalline matter is quickly recognized. An objection to the use of these mountants is that tests with ordinary aqueous reagents cannot be applied to such preparations; but this does not detract from their usefulness for a preliminary survey of a number of specimens. Chemical tests can be carried out in other ways, probably not microscopically.

It is a useful practice also to examine cattle and chicken foods in a similar way. Under these conditions material like Soya flour (see Fig. 23), which contains numerous small but characteristic crystals of calcium oxalate, and poisonous substances like hellebore, i.e. *Veratrum* (containing bundles of acicular calcium oxalate crystals), may be identified by the ease with which the crystals become evident, especially if polarized light is used.

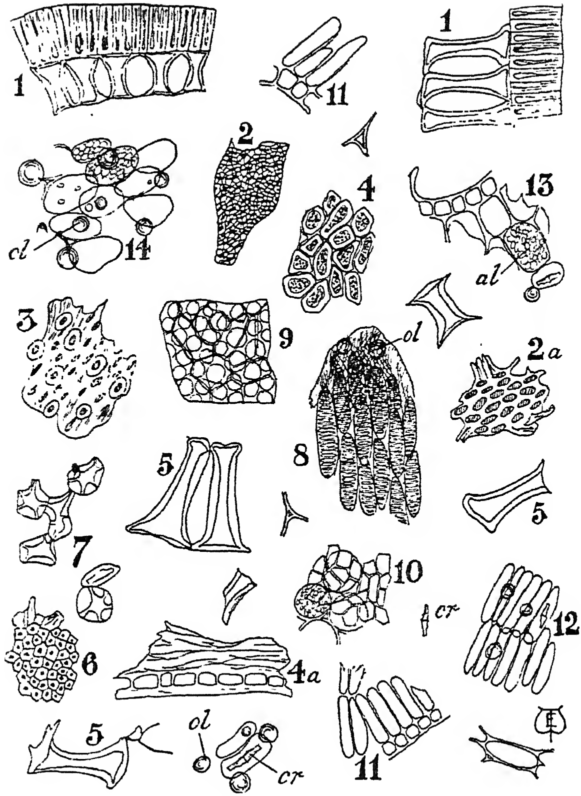

Fig. 23.—Powder of Soya bean, *Glycine hispida*, Maxim.

(1) Palisade epidermis and bearer cells in transverse section. (2) Palisade epidermis in surface view from above. (2*a*) The same from below. (3) Palisade epidermis and bearer cells in surface view. (4) Aleurone layer within seed-coat in surface view. (4*a*) The same with compressed parenchyma in section. (5) Isolated bearer cells and portions of such cells. (6) Upper layer of double epidermis from hilum, in surface view. (7) Modified parenchyma and sclerenchyma from region of hilum. (8) Tracheids from hilum-furrow. (9) Epidermis and underlying cells from flat face of cotyledon. (10) Epidermis and underlying cells from rounded face of cotyledon. (11) Epidermis of flat face and palisade cells of cotyledon in transverse section. (12) Palisade cells from cotyledon. (13) Epidermis and cells of mesophyll from rounded face of cotyledon. (14) Cells of mesophyll of cotyledon; *al.* aleurone grains; *ol.* oil globule; *cr.* calcium oxalate crystals. All × 150.

CHAPTER VI

MORE VIGOROUS METHODS OF PRELIMINARY TREATMENT

Crude Fibre. More vigorous methods of treatment are frequently used for the destruction of cell contents and certain other constituents of substances, resulting in a concentration of the residual and more highly resistant particles which are often also very characteristic in appearance, and particularly useful for purposes of identification. Two of the most important and most largely used of these processes are the preparation of crude fibre and the making of a wet ash.

For the examination of all kinds of starchy materials, like flour, pepper (see Fig. 38, p. 112), ginger, and many drugs, as also for dark-coloured substances like coffee and chicory, one should prepare a crude fibre with a view to obtaining the cellular structures in a good condition for microscopic observation. Two largely-used methods are employed for this purpose, both of which involve alternate digestion with boiling acid and alkali. The treatment with acid converts starch to glucose or oxalic acid, which is removed in the washings, while proteid and much dark colouring matter is dissolved and removed by the action of the alkali and the subsequent washing.

The more rapid process, known as the "Dutch method," consists in boiling about 2 gms. of the powdered substance in a 100 c.c. porcelain dish with 50 c.c. of 10 per cent. nitric acid for thirty seconds, and straining out the residue by filtration at the pump through a fine-meshed cloth (Horrockses' longcloth M2 is very suitable). After washing

well with about 100 c.c. of boiling water, the material is removed from the cloth and again boiled in the dish for thirty seconds with 50 c.c. of a 2·5 per cent. caustic soda solution. The fibre is strained out and washed at the pump, and a small quantity is mounted for microscopical examination. The damp fibre is easily and completely removed from the cloth used for the filtrations by stretching the material tightly, like the membrane of a drum, over the rim of a watch-glass and scraping off the fibre by means of a flexible spatula.

The method most commonly used in this country and in America is to boil in succession with diluted sulphuric acid and solution of caustic soda. The strength adopted for the solutions is 2 per cent. in Great Britain or 1·25 per cent. in America. About 2 gms. of the powder is boiled for half an hour with 125 c.c. of 2 per cent. or 200 c.c. of 1·25 per cent. sulphuric acid in a conical flask. The decoction is strained through a piece of fine cloth and washed with hot water at the pump; then it is boiled in a similar way with the caustic soda solution, strained, and washed again. It is a good plan to complete the process by a final treatment with acid, followed by straining and washing.

Whichever method is adopted, it is necessary to remove, by a preliminary treatment with a suitable solvent, the greater part of the fat or oil present in such materials as cocoa and mustard. (See above, p. 60, "Use of Solvents for Fats and Oils.")

Coffee and Chicory. The accompanying figures show the characteristic features of the fibres yielded by coffee (Fig. 24) and chicory (Fig. 25). The large, pitted woody elements and the laticiferous vessels make it an easy matter to identify chicory when mixed with coffee, which consists chiefly of the powdered endosperm, composed of isodiametric parenchyma with large oval pits. The woody tissues normally present in coffee are the sclerenchymatous cells of the seed-coat and the vessels from the raphe, neither of which resemble the woody elements of chicory.

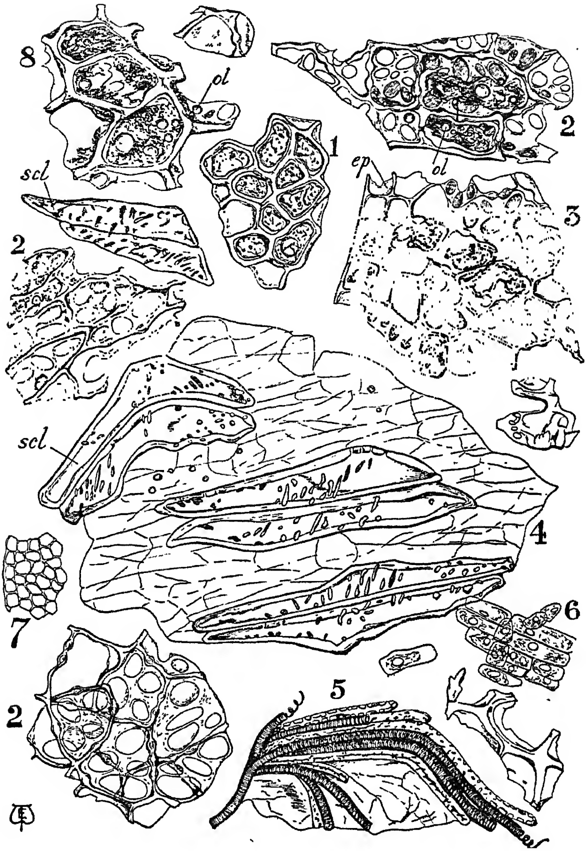

FIG. 24.—Powdered Coffee, *Coffea arabica*, Linn.

(1) Epidermis of endosperm in surface view. (2) Fragments of endosperm, showing isodiametric parenchyma with oval pits and drops of oil, *ol*. (3) Piece of endosperm, including epidermis *ep*, as seen in section. (4) Seed coat with sclerenchymatous cells, *scl*. (5) Portion of seed coat with vessels from the raphe. (6) Cells from radicle of embryo. (7) Part of a cotyledon. (8) Fragment of endosperm. All × 200.

If one wishes to preserve a crude fibre for reference it should be put into a small bottle with some water and a

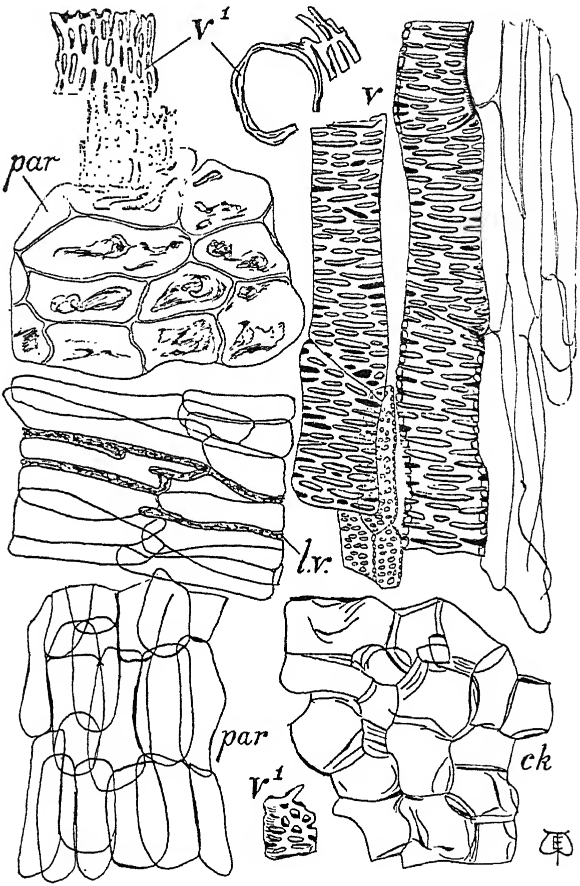

FIG. 25.—Powdered Chicory, *Cichorium Intybus*, Linn.

v, Wood vessels; *v*¹, Fragments of vessels; *lv*, laticiferous vessel; *par*, parenchyma; *ck*, cork. All × 200.

little formaldehyde, when it will keep in good condition for an indefinite length of time.

Use of Nitric Acid. Nitric acid of sp. gr. 1·42 diluted to five times its volume by the addition of water is a useful reagent for the separation of the epidermis of leaves and stems. The leaf is cut up into pieces about $\frac{1}{4}$ in. (6 mm.) square, or a stem is cut into $\frac{1}{4}$-in. lengths, and the material is boiled gently with the diluted acid until the epidermis can be separated readily. This is tested by removing a portion and endeavouring to strip off the epidermis upon a slide; when this can be easily accomplished, the action is stopped by pouring the contents of the test-tube into water in a beaker. The epidermis from both fresh and dried leaves can be separated by this process, which is in many ways preferable to the use of potash or soda, because the acid produces less swelling and distortion of the walls.

This diluted nitric acid gives very good results as a fluid for softening hard woody tissues, such as olive stones and coco-nut shells. To bring such refractory structures into a condition fit for cutting sections one boils them with the dilute acid for a few minutes, and then allows the material to macerate in the acid at a gentle heat until a portion removed and washed with water is found to cut with sufficient ease; this will take two or three hours. When finished, soak in several changes of distilled water and reserve for sectioning.

The softening action of hydrofluoric acid is less destructive to the tissues than that of nitric acid, and this acid is therefore more commonly employed; details are given below (see p. 71).

Schulze's Maceration Fluid. In conjunction with potassium chlorate, nitric acid diluted with an equal volume of water forms the liquid known as Schulze's Maceration Fluid, and is used to disintegrate hard woody substances such as olive stones and other nut shells and pieces of wood. The fluid dissolves the substance of the middle lamella, and the pieces of macerated tissue can then be broken up into their constituent cells by teazing them out in water with needles. The best way to carry out the

operation is to cut the material into small pieces and heat it in a flask to the boiling-point with the diluted nitric acid, then add a little powdered potassium chlorate, and allow the reaction to proceed, warming gently, if necessary, to maintain a slight effervescence. Fresh quantities of powdered potassium chlorate are added as needed. When the woody tissue appears to be almost entirely bleached, and shows a tendency to fall to pieces, it should be tested by pressing a piece with a glass rod to see if it can be broken up by a slight pressure, and should this be the case, the action is stopped by pouring the contents of the flask into distilled water. Allow the material to settle, and decant off the acid liquid; add a fresh quantity of distilled water, allow to soak for a time, and decant again, repeating the process until the acidity is removed. The material can now be broken up on the slide and examined microscopically.

Chromic and Nitric Acids. Another method of isolating cells, which injures them less than the chlorate method, is to steep the material in an aqueous solution containing 10 per cent. of nitric acid and 10 per cent. of chromic acid (chromic anhydride). The action is continued until a piece of the material can be easily broken up with needles upon the slide. One may hasten the action by warming, but better results are produced in the cold. Sulphuric acid is sometimes used in place of the nitric acid, and gives a reagent which acts rather more rapidly and is often preferred.

In some instances this mixture of acids may be usefully applied to fairly thick sections, which are mounted in the reagent and, after standing for a suitable length of time, the cells are separated by gentle pressure upon and a sliding movement of the cover-glass.

Hydrofluoric Acid. Olive stones, coco-nut shells, peach stones and hard woods are commonly softened for sectioning by maceration in commercial hydrofluoric acid. This acid softens the cell walls by dissolving out any

mineral matters and silica that may be present, but it does not attack the middle lamella, so that the cells of the softened tissues do not fall apart.

In a general way, the woody structures are first thoroughly boiled with water until all the air has been driven out. After cooling, the pieces are transferred to the commercial hydrofluoric acid and allowed to soak until, after washing with water, they are found to cut easily with a knife. This will usually take a week or two, and if the material is still too hard it must be macerated for a further period until sufficiently soft to cut easily; the maceration may need to be continued for as long as six weeks and the acid may need to be changed for fresh during the process.

The pieces, after thorough washing in several changes of water, are stored in a mixture of equal parts of glycerine and 30 per cent. alcohol, and after a week or so they will be in a condition suitable for sectioning.

The maceration in hydrofluoric acid must be carried out in dishes made of paraffin wax or in glass dishes coated all over with wax. Great care must be taken, in using the acid, to avoid getting any on the fingers, as it quickly makes very troublesome burns. No pain is felt at the time, but after a while much pain and swelling result and sores difficult to heal are ultimately produced.

Hydrochloric Acid. Hydrochloric acid of specific gravity 1·16 diluted by the addition of four times its volume of water is a valuable reagent for the removal of starch from powders. For example, one may wish to isolate mites, such as *Aleurobius farinæ*, from a specimen of infested flour. For this purpose one boils 2 to 5 gms. of the flour with about 100 c.c. of the acid until hydrolysis of the starch is complete. The liquid is then centrifuged and the deposit washed by pouring off the supernatant liquid and centrifuging a few times with water, after which the deposit is shaken up with 5 per cent. caustic soda solution, centrifuged again and washed several times with water. The

final deposit consists almost entirely of mites, which may be permanently mounted by placing a drop on a slide, adding a drop of glycerin jelly and a cover-glass.

Disintegration by Caustic Alkali. Caustic soda or potash in 2 to 5 per cent. aqueous solution is a reagent having a solvent action upon starch and proteids and also a strong softening and disintegrating effect upon cellulose. Lignified and cuticularized walls are much less readily attacked, and hence this reagent is used to isolate such elements when they occur in association with cellulose tissues and with starch and proteids. Barks (which in the commercial sense consist mainly of phlœm tissues), seeds, and leaves, are the most important plant organs which exhibit such characteristics, and it is for the isolation of the more resistant parts, viz., fibres, sclerenchyma, laticiferous tissue and cuticles, of such structures that caustic alkali is most largely employed. One must bear in mind the swelling action of the reagent, which is most disturbingly evident in the case of a leaf epidermis (see above for remarks upon the use of nitric acid).

The material to be treated is cut up into small pieces and digested in a beaker flask with some of the alkali on a water-bath until a portion removed and dissected upon a slide shows that the action has been carried far enough. When this point has been reached, the liquor is decanted and the softened material is washed by steeping it several times in fresh quantities of distilled water so as to remove the alkali.

Caustic soda or potash has a strong solvent action upon all kinds of animal structures, and is useful to dissolve away much of the softer parts of insects and to soften and modify the chitin in such a way that the structures can be later subjected to the action of strong clearing reagents like turpentine and clove oil without undergoing movement or shrinkage. In such cases, after washing with water, any remaining traces of alkali and water are removed by subsequent treatment with glacial acetic acid.

Wet Ash. The preparation of a wet ash is particularly useful for determining the presence of agar-agar jelly in jams, jujubes, and other similar products. From such commodities one can isolate the diatoms and sponge spicules by the following process :—Treat from 5 to 10 gms. of the jam on a water-bath with about 40 c.c. of diluted hydrochloric acid (one volume of acid and two volumes of water) until the jelly is destroyed. Filter through a small filter-paper (about 5·5 cm. in diameter), and wash two or three times with water. Allow to drain; fold up the paper with the residue of the jam, and allow it to fall into 20 c.c. of strong sulphuric acid in a hard glass digestion flask; add 10 gms. of potassium sulphate or anhydrous sodium sulphate, and heat until colourless, as in the process for the estimation of nitrogen. When colourless, cool, add water, and separate the siliceous matter by centrifuging the liquid. The residue consists almost entirely of sponge spicules and diatoms, which can be transferred to a slide by a dipping tube and identified microscopically. Other products are treated in a similar way, with such slight modifications as will readily suggest themselves.

Agar-Agar. A standard specimen of diatoms and spicules from agar-agar can be prepared by using about 2 gms. of agar and proceeding as directed above. The accompanying figure (Fig. 26) shows the diatoms and sponge spicules obtained from a specimen of ordinary strip agar.

Permanent preparations are made by decanting the water from the deposit, and washing two or three times by decantation or centrifugation with alcohol. A little of the deposit is removed by a dipping tube, placed on a slide, mixed with a drop of amyl-sandarac (sandarac, 32 gms.; castor oil, 4 c.c.; amylic alcohol, 64 c.c.; see Wallis, "The Use of Amylic Alcohol and Sandarac in Microscopy," *Journ. Quekett Micro. Club*, April 14, 1919, p. 13), and exposed to the air to allow the bulk of the alcohols to evaporate, after which the cover-glass is applied. If one

prefers to mount in Canada balsam, the alcohol is drained off, and the residue dried by heating in a steam oven. To the dry deposit add turpentine, and boil. A small drop of the turpentine with some of the flinty particles is transferred to a slide, a drop of xylol-balsam added, and a cover-glass applied.

Although one has found diatoms in jams on several occasions, *Arachnoidiscus* is usually absent. This fact would seem to indicate that the filtration or clarification to which the jelly is subjected results in the removal of the large diatoms, while the smaller ones pass through the

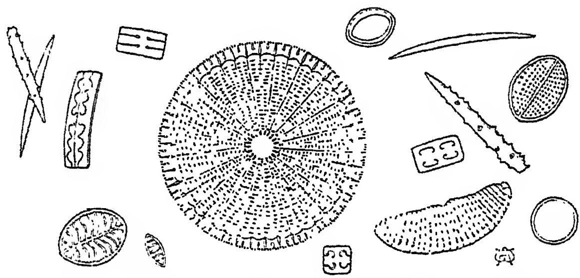

FIG. 26.—Diatoms and Sponge Spicules from a specimen of Agar-agar.

× 200. The large discoid diatom is *Arachnoidiscus Ehrenbergii*. Two sponge spicules are seen lying one over the other on the extreme left of the drawing, and two others, separate, on the right.

filtering medium. To test this conclusion I prepared an agar-agar jelly and filtered it through a piece of flannel. A wet ash was made both from the filtrate and also from the portion left on the flannel. A microscopical examination of the siliceous residue from the wet ash showed, in the case of the filtrate, large numbers of sponge spicules and small diatoms, with a complete absence of *Arachnoidiscus*, while numerous specimens of this diatom were found in the ash from the portion retained by the flannel. This experiment shows that filtration of jam through flannel or felt would result in the removal of discoid diatoms of the *Arachnoidiscus* type.

It has been stated by Parkes (*Analyst*, 1921, p. 239) that diatomaceous earth is sometimes used for the clarification of jams and that diatoms may be present from this source. Hence care must be exercised in concluding that the finding of diatoms indicates the presence of agar jelly. The result should be confirmed by carrying out chemical work upon the gelatinizing power of the jelly.

CHAPTER VII

MICROCHEMICAL TESTING

Scope of Employment. The chemical reagents which are of real service in the regular course of analytical microscopy are quite limited in number. Among the more important are those termed clearing reagents, several of which have already been considered. Other substances serve as tests for certain widely distributed cell contents which are identified either by undergoing solution in the test liquid in a characteristic manner or by acquiring some distinguishing colour. Reagents which produce precipitates, although useful in particular instances, and especially in the examination of sections of tissues, are not so generally serviceable in analytical investigations.

Microchemical testing, in a general sense, carried out on the lines of ordinary qualitative chemical analysis, is only to be recommended in very special circumstances. Such work is not always to be depended upon to give a final and complete answer as to the identity of the substance that is being examined. It is useful when the amount of available material is very small and under circumstances where the number of possibilities makes identification in the ordinary way hopeless unless one can test boldly with a definite object in view, as is the case when a preliminary indication has been obtained microscopically. The microscope is also properly employed in chemical analysis to ascertain the crystalline or amorphous nature of a sublimate or precipitate or of a residue left after the evaporation of a solvent. Used in this way the microscope is of the greatest value as an aid to ordinary chemical analysis, especially in

toxicological investigations and in other instances where only minute quantities of material are available.

Application of Reagents. There are two principal methods of applying reagents, viz. :—(1) To mount the object in the reagent just as one would in water, and (2) to irrigate the specimen with the test liquid by drawing it across the preparation beneath the cover-glass by means of a piece of blotting paper. For this purpose a drop of the reagent is placed on the slide against the edge of the cover-glass while the blotting paper is applied to the opposite side, thus drawing a stream of liquid across the preparation. The advantage of the irrigation method is that one can watch through the microscope the whole progress of the reaction, and one thus obtains a clearer idea as to the appearance of the particles both before and after the application of the test. In the process of irrigation, however, the reagent becomes diluted in its passage beneath the cover-glass, and should therefore be applied in a more concentrated form than when the object is mounted directly in the test liquid. For instance, a twentieth-normal solution of iodine is most suitable for irrigation, while one that is ten times weaker is more suitable for use as a mountant. Similarly, a stronger liquid is needed for the irrigation or treatment of sections than for testing particles that are not enclosed within cell walls. For example, a 33 per cent. acetic acid should be used for testing calcium carbonate in the form of cystoliths, but a 10 per cent. solution is quite strong enough for testing foraminifera or isolated crystals such as occasionally occur in water deposits.

A reagent may often be applied by a method similar to irrigation without the use of blotting paper, which is apt to cause too strong a flow of fluid across the slide, and sometimes produces an inconvenient disturbance of the particles under observation. This is effected by having a minimum amount of water under the cover-glass against the edge of which a drop of reagent is placed. The drop immediately

begins to flow underneath by capillary attraction. By manipulating the slide under the microscope one is able to watch the approach of the liquid to any special particles and to observe all the changes which occur as the proportion of reagent present gradually increases.

In making all such experiments great care must be taken to avoid injury to the microscope—both the objectives and the stage. Generally speaking, low power objectives (not less than $\frac{2}{3}$ in. or 17 mm. focus) give sufficient magnification for this kind of work, and their use not only lessens risk of damage to the instrument but also gives a considerable working distance between the objective and the slide, resulting in greater freedom for manipulations upon the microscope stage. The use of objectives having a focus as short as $\frac{1}{6}$ in. or 4 mm. is necessary in certain instances only for the examination of products resulting from the reactions carried out upon the slide, so that the experiment can be made under a low power lens and higher powers are used subsequently if necessary.

Iodine Water. Iodine is the most important of all the reagents used for microchemical testing. A suitable solution is obtained by diluting 5 c.c. of decinormal iodine to 100 c.c. with distilled water, or, for use by irrigation, the decinormal volumetric solution should be diluted with an equal volume of water. Iodine colours starch blue and imparts a yellow colour to aleurone, skin flakes, and animal matter in general.

When examining water deposits a portion should be mounted and irrigated with iodine solution, which throws into prominence starch grains, to the occurrence of which reference has already been made (see p. 35), and also has the effect of slowing the movement of infusorians, and so making them more easily identifiable. Eventually infusorians, rotifers, and similar organisms contract and die, and are stained yellow. Waters sometimes contain skin flakes and other animal products, such as fibres of wool, all of which are stained yellow by the iodine.

When such materials are present in abundance they indicate contamination by slop water, since water in which the hands have been washed contains these substances in large quantity.

Iodine is valuable for the identification of apple pulp in jams. The test made under the microscope is far more certain and more delicate than that usually recommended of treating the decolorized jam with iodine in a test-tube. It is best to place a small piece of the jam pulp upon a slide with the point of a knife and to mix it with a little iodine water, then apply a cover-glass and examine. Some of the large rounded apple-pulp cells invariably show blue-stained contents. The conclusion may be confirmed by searching the washed jam tissues for pieces of apple core as already suggested above (see p. 53).

The deposit from solution of sugars (see p. 53), will also repay treatment with iodine, which stains starch grains and skin flakes, which are often present when the sugar has been unnecessarily exposed to the dust of the shop and to excessive handling.

Acetic Acid. Acetic acid is most frequently employed for the recognition of calcium carbonate, and its distinction from calcium oxalate. The test is useful for all water deposits containing crystalline matter or shells of foraminifera. A preliminary indication of chalky foraminifera is obtained by examining the deposit by reflected light, all light from below the stage being cut off by interposing the hand between the mirror and the stage. Under these conditions the foraminifera (see Fig. 27) appear as white, rounded, and spirally twisted masses, which are then further tested by irrigation with 10 per cent. acetic acid, which dissolves them with a simultaneous evolution of gas. It is interesting to note that the bubbles of gas frequently do not form upon the chalky particles themselves, but gradually collect in the liquid at a short distance away.

A useful way of preparing microscopic crystals of calcium carbonate for studying the action of acetic acid upon this

substance under the microscope is to pass carbon dioxide gas through water in which some precipitated chalk is suspended. On boiling the filtered solution one obtains a deposit of small crystals of calcium carbonate well suited for an experimental microscopical study of the action of acetic acid upon crystalline carbonates.

Blood Test. For the identification of blood one mounts a fragment of the dried blood with glacial acetic

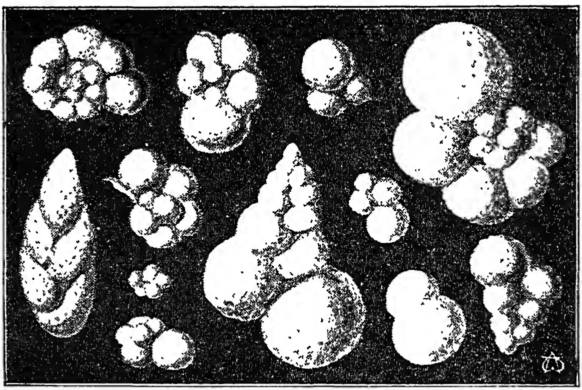

FIG. 27.—Cretaceous Foraminifera from a Water Deposit.

× 200. They are shown as seen by reflected light. The forms represented include specimens of *Globigerina* and *Textularia*.

acid and boils it gently. On cooling and examining with the microscope, dark red crystals of hæmin (known also as Teichmann's crystals), are seen under the cover-glass. If the blood is fairly fresh glacial acetic acid alone is sufficient, but if the stain is an old one, a small crystal of sodium chloride is added as well as the acid and then the mixture is boiled.

Hydrochloric Acid. Hydrochloric acid of specific gravity 1·16 is useful as a clearing reagent because of its strong solvent powers; it destroys starch and dissolves

both calcium oxalate and calcium carbonate, the former without evolution of gas. Calcium oxalate is insoluble in acetic acid, which fact, together with its solubility in hydrochloric acid, serves for the identification of this substance.

Vegetable sections treated with hydrochloric acid are cleared of starch and many other cell contents, and nuclei become clearly defined.

In connection with the examination of water deposits, hydrochloric acid yields valuable information. When a red-brown amorphous-looking material is present it is often important to determine its nature, and this can be done by noting the action of hydrochloric acid upon it. The experiment may be carried out on the slide, or a quantity of the deposit may be transferred by means of a dipping tube to a test-tube, and gently warmed with strong hydrochloric acid. The clarified deposit is allowed to settle, and a small amount is removed and examined in the usual way. The red-brown matter may be iron rust or fine red earth or peaty material. If it is iron rust, there will be an entire absence of residue, or a little indefinite amorphous matter may be left. When the deposit is red earth, the residue consists of nearly colourless particles of sand and very fine earthy matter (clay), and when peat is present the brown colour is not destroyed, and the deposit has much the same appearance as before its treatment with hydrochloric acid.

Caustic Soda Solution. A 50 per cent. solution of soda or potash is used as a test for suberized cell walls, which become yellow when mounted in the reagent, and on boiling they are replaced by yellow oily drops.

Caustic soda in 10 per cent. solution is an important microchemical reagent. It dissolves starch and aleurone, and causes more or less swelling of most vegetable tissues. It is therefore often used as a clearing reagent, although for general purposes chloral hydrate is to be preferred. It is also used in the examination of oil-

cakes, cattle and poultry foods to aid in the identification of pieces of seed coats picked out from them by hand. Such portions of seed coats are macerated in the alkali to render separable the various layers of cells, so that their structure may be studied and compared with similar preparations from authentic materials. The fragments of seed coats are covered by the soda solution and allowed to soak for about an hour, after which they are thoroughly broken up by teazing them out with needles; dilute glycerin is then added and a cover-glass. The action of the alkali may be hastened by heating the fragments with the solution in a beaker-flask on a water-bath until a piece taken out is found to be sufficiently softened to break up easily.

A common ingredient of mixed cakes and poultry spices is the carob, or locust bean, the fruit of *Ceratonia Siliqua*, L., N.O. Leguminosæ, which is also stated to be added in quantity to inferior chocolate. The presence of these beans can be established by moistening a little of the powder upon the slide with alcohol and mounting it in caustic soda solution; this gives a greenish-blue coloration to the remarkable brown, amorphous bodies, sometimes termed "inclusions" or "wrinkled bodies," found in the cells of the husk of the pod (see Fig. 28). The blue colour gradually deepens in tint and changes on long standing to purple, and finally, after a lapse of twelve to twenty-four hours, becomes red-brown. These wrinkled bodies are insoluble in water, alcohol and glycerin, and in acetic, hydrochloric, and dilute sulphuric acids. Similar structures have been found in certain other plants, such as tamarinds, dates, and the leaves of *Rhamnus cathartica*.

Hesperidin is another substance identifiable by caustic alkali. It occurs in crystals and amorphous masses in orange peel, buchu leaves, hemlock (*Conium*) and other plants; it is insoluble in water, alcohol, chloral hydrate, strong hydrochloric acid, nitric acid, and diluted sulphuric

acid, but soluble with the production of a yellow colour in solution of caustic soda or potash.

Dilute caustic soda, which is without action upon vegetable fibres, will dissolve animal fibres such as sheep's wool and is a good reagent to aid in their identification. The material is mounted in 5 per cent. aqueous caustic soda or potash and the preparation is gently boiled, when animal fibres dissolve entirely.

Picric Acid. A saturated solution of picric acid in

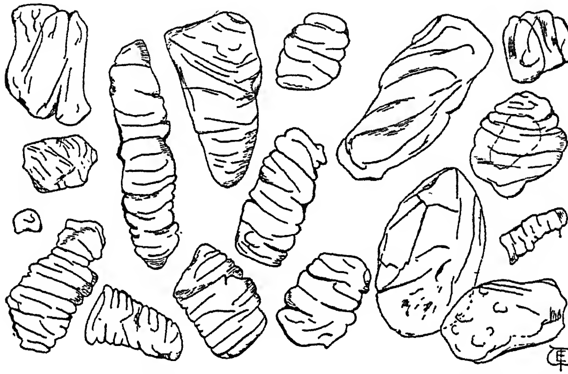

FIG. 28.—Irregular, Brown, Amorphous Bodies from the Husk of the Carob Bean, *Ceratonia Siliqua*.
× 100.

water (soluble 1 in 50) will stain animal fibres yellow, while cellulose fibres remain uncoloured. This reaction is very useful for examining mixed materials such as gray filter papers or bandage materials such as "domette" bandage.

To search for wool or animal fibres in a gray filter paper, take about a square inch of the paper, tear it up into very small pieces and put it into a test-tube about one-third filled with water; shake vigorously until a uniform pulp is produced. Remove a small piece of the pulp on the point of a needle, mount it in saturated aqueous solution of picric acid and allow to stand for five or ten minutes,

wash the fibre two or three times by irrigation with distilled water and examine the preparation microscopically when all the animal fibres are clearly distinguished by their bright yellow colour. The precise identity of the fibres present can only be known by comparison with good drawings and with standard preparations of authentic fibres. The accompanying figure shows the appearance of sheep's wool under the microscope.

In a similar way one can examine the fibres in the warp and the weft of a piece of domette bandage. About a

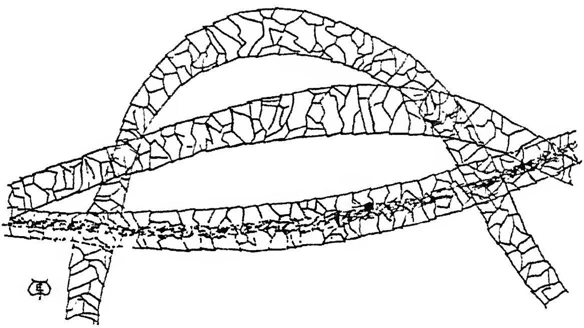

FIG. 29.—Fibres of Sheep's Wool.

$\frac{1}{4}$ in. is cut off from a thread of the warp and of the weft; each is teazed out separately upon a glass slide and mounted in picric acid solution. After allowing the mount to stand for a few minutes, wash by irrigation with water and examine microscopically. The fibres of the weft, i.e. those threads which run through the length of the bandage, should be entirely of wool, and those of the warp, i.e. those threads running transversely, entirely of cotton (see Fig. 31, p. 89, for a drawing of cotton fibres).

Sulphuric Acid. Strong sulphuric acid of specific gravity 1·843 is the best reagent for the identification of suberized tissues. The section is mounted in the acid,

which gradually dissolves away all the structures except those that are suberized, so that the cuticle and any other corky walls present are the only parts that remain after the preparation has stood for some time. Cotton hairs are also completely dissolved by an acid consisting of eighty volumes of strong sulphuric acid and twenty volumes of water.

In conjunction with iodine, a fairly strong sulphuric acid forms an excellent test for cellulose. The acid should be made by mixing two volumes of the strong acid (s.g. 1·843) with one volume of water, and the iodine is conveniently prepared by diluting one volume of decinormal iodine to five volumes with distilled water. The section is mounted without a cover-glass in a drop of the iodine solution, and after a few seconds the iodine is removed by blotting paper; a drop of the sulphuric acid is now applied and a cover-glass. Under these conditions the cellulose walls swell up and are stained blue.

Calcium Oxalate. A weaker solution of sulphuric acid is used as a test for calcium. A suitable reagent is obtained by diluting one volume of the strong acid to ten volumes with distilled water. This test may be applied to the solution of calcium carbonate in acetic acid, when radiating groups of needle-shaped and flat rhomboid crystals of calcium sulphate gradually form in the liquid. Crystals of calcium oxalate present in plant sections can be tested by mounting them in acid of the same strength, but the reaction takes place rather slowly, so that it is better to use an acid made by diluting one volume of strong acid to five volumes with water; this gives a reaction in a few minutes (see Fig. 30).

To gain confidence in the use of this test, and for purposes of comparison, one will need a specimen of micro-crystalline calcium oxalate. Such crystals can be obtained by two methods. The first method is to prepare equal volumes of 0·5 per cent. solutions of oxalic acid and calcium chloride, raise both to the boil, and very gradually, especially at

first, add the calcium chloride to the oxalic acid (the mixing of 500 c.c. of each should take about an hour). Allow to stand until cold, filter out, and wash the precipitated calcium oxalate, which will be in well-formed micro-crystalline prisms (see Fig. 30). A second method is to pre-

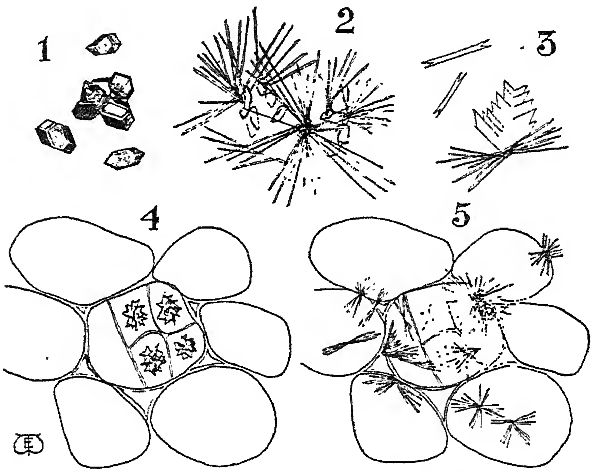

FIG. 30.—Drawings to Illustrate the Action of Sulphuric Acid upon Calcium Salts.

All × 200. (1) Prismatic crystals of calcium oxalate prepared by mixing 0·5 per cent. solutions of calcium chloride and oxalic acid. (2) Needle-shaped crystals of calcium sulphate, produced by the action of 20 per cent. (by vol.) sulphuric acid upon artificial calcium oxalate. (3) Calcium sulphate crystals produced by irrigating a solution of calcium carbonate in acetic acid with 10 per cent. (by vol.) sulphuric acid. (4) Part of a section of a petiole of ivy (*Hedera Helix*), showing four cluster crystals of calcium oxalate. (5) The same section 15 minutes after treatment with 20 per cent. (by vol.) sulphuric acid. A few dots represent two of the rapidly disappearing cluster crystals. It will be noticed that the calcium sulphate needles do not necessarily form in the position previously occupied by the oxalate.

cipitate calcium oxalate in the ordinary way by adding excess of powdered solid ammonium oxalate to nearly boiling calcium chloride solution. Filter out the precipitate, which is almost amorphous, and wash it. Suspend the precipitate in water, warm it, and gradually add nitric acid until complete solution results: then pour into cold water and allow to stand until the calcium oxalate has

crystallized out. This process yields good-sized and well-formed microscopic prisms of calcium oxalate for use as a standard of reference.

Phloroglucin. Phloroglucin is the reagent which gives the most distinctive reaction with lignified tissues. It is not only useful for staining sections, but also for vegetable powders and for water deposits. When examining powders a small quantity is mixed with a few drops of a 1 per cent. solution of phloroglucin in alcohol, and allowed to become nearly dry by evaporation of the alcohol; strong hydrochloric acid (s.g. 1·16) is then added and a cover-glass applied; all woody structures, such as ground olive stones and coconut shells, are stained red. An aqueous 1 per cent. solution of phloroglucin answers equally well, but it is then necessary to dry the powder mixed with the solution by heating the slide in a steam oven before adding the hydrochloric acid. Other acids than hydrochloric give this reaction equally well, and one may substitute a sulphuric acid made by diluting one volume of strong acid to five with water, or a slightly diluted nitric acid. Hydrochloric acid is generally preferred because it is less likely to yield insoluble products which might obscure other important features.

A deposit from a water is treated by placing a small amount upon a glass slide by means of a dipping tube and drying it in the oven at 95° to 100° C., after which the phloroglucin and hydrochloric acid are added as described above. In this way deposits from well waters are examined for dust from decaying wooden beams or from wooden covers; such fragments of rotten wood are frequently found in association with septate fungal spores, while the walls of such wood cells are themselves often much eroded.

Phloroglucin is also used for the detection of mechanical wood pulp in paper. For this purpose the paper is pulped as described above for filter papers under Picric Acid, p. 84. A small piece of the pulp is placed on a slide, water is removed as much as possible, phloroglucin solution is added and allowed to evaporate nearly to dryness; strong

hydrochloric acid is now applied and a cover-glass. Woody fibres are coloured red and can be rapidly picked out and identified microscopically.

Cuoxam. Cuoxam is an ammoniacal solution of copper

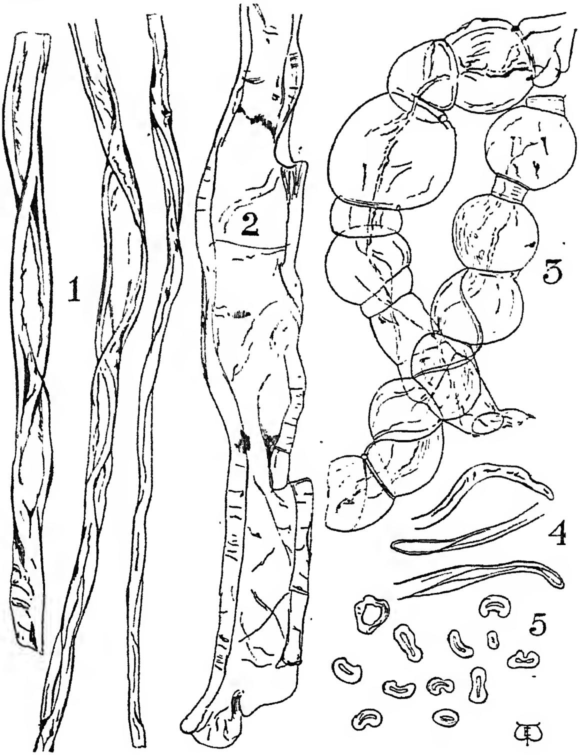

FIG. 31.—Cotton—Hairs from the Seeds of *Gossypium sp.*

(1) Hairs mounted in dilute glycerin. (2) Absorbent cotton treated with ammoniacal copper oxide. (3) Raw cotton treated with ammoniacal copper oxide. (4) Tips of cotton hairs. (5) Transverse sections of cotton hairs. All × 200.

oxide and is best prepared by rubbing down 0·5 gm. of commercial copper carbonate to a smooth cream with 10 c.c. of distilled water in a mortar and then adding 10 c.c.

of strong solution of ammonia (s.g. 0·880). The reagent thus prepared is a clear deep blue liquid.

This fluid dissolves cellulose walls, but is without solvent action upon woody or suberized walls, or upon animal fibres. It is useful for the identification of fibres of cotton and linen, which rapidly swell and finally dissolve in the reagent. The above figure (Fig. 31) shows hairs of cotton in their normal condition and also swollen by the action of cuoxam. It will be noticed how differently many of the hairs of raw cotton behave; instead of swelling uniformly like hairs of absorbent cotton wool, they swell up into balloon-like portions, separated by constricting bands. This appearance is due to the presence of a cuticle upon the raw fibres, while cotton that has been treated with dilute caustic soda to render it absorbent, is destitute of a cuticle and consequently swells uniformly.

Other Reagents. All the reagents hitherto considered have a wide range of application which gives them special importance in microchemical work. There are other reagents used for the identification of cell contents, such as ruthenium red for mucilage (see p. 106), Millon's reagent for aleurone, Braemer's reagent for tannin,. tincture of alkanna for oil, alkaloidal reagents, chlorzinciodine, and others, but they are less generally used in analytical microscopy. The composition and use of these reagents is fully described in Greenish's *Microscopical Examination of Foods and Drugs.*

CHAPTER VIII

PHARMACEUTICAL AND TOXICOLOGICAL MICROCHEMISTRY

The materials used in pharmaceutical practice include those that are met with in toxicological investigations, so that analytical work in these two departments requires the application of the same tests, although sometimes with different objects as an ultimate aim. In the majority of such inquiries the amount of material available for analysis is strictly limited, and a preliminary microscopical investigation of the chemical behaviour of a very small portion of the specimen, or of a purified product obtained from it, will give valuable guidance for the direction of the main analysis upon the bulk of the material. The possibility of obtaining a conclusive and accurate result is thus greatly facilitated by a skilful and intelligent use of microscopical methods.

Chemicals having a Definite Microscopical Structure. In addition to organized drugs and their products, there are a few substances used in pharmacy, and frequently occurring in analytical practice, which have a definite microscopical structure. Among these may be mentioned precipitated and sublimed sulphur, kieselguhr, talc or French chalk, and precipitated chalk. Of these substances kieselguhr has an organized structure, being composed entirely of the siliceous valves of diatoms. This substance, which is identified chemically as silica, can only be definitely recognized as a diatomaceous earth by means of the microscope, which will show characteristically sculptured valves similar to those represented on Plate A (see p. 38).

Flowers of Sulphur. In connection with sulphur it will be noted that all the official medicinal preparations with the exception of the ointment are now made from precipitated sulphur. It might appear, at first sight, to be a matter of indifference as to which variety of sulphur is used, but the precipitated sulphur being in a much finer state of subdivision, and containing less angular particles, is more suited for internal administration owing to its more ready conversion into other compounds in the alimentary tract, and to its less irritant property. For use as an ointment, the softness of precipitated sulphur is very detrimental to its efficacy as a cure for scabies. In this case the more gritty sublimed sulphur must be used, and partly owes its remedial power to the scarifying action of the sharp-edged particles in opening up the burrows in the skin, at the extremities of which the acarids causing the disease are to be found. It thus becomes a matter of importance to be able to distinguish between the varieties of sulphur, and although this cannot be done by chemical analysis, it is accomplished with ease and certainty by the microscope.

Sulphur is most conveniently examined by mounting a small amount in cresol or clove oil, which quickly displaces all the air from the powder, and comes into close contact with the sulphur particles, so that a sharply defined image is produced in the microscope. Flowers of sulphur occurs in the form of spheroidal masses varying in diameter from 5μ to 40μ and agglutinated together in irregular groups as shown in Fig. 32 (1). In some specimens of sublimed sulphur there also occur irregular transparent semi-crystalline lumps produced as a result of a somewhat higher temperature in the cooling chamber, in which the sublimed sulphur is collected. A variety of commercial sulphur, sometimes offered as sublimed sulphur, is obtained by powdering roll sulphur. This powder is easily distinguished from the official sublimed sulphur because it is composed entirely of small angular particles; there is a

complete absence of the characteristic spheroidal masses found in the genuine article, see Fig. 32 (3).

Precipitated Sulphur and Calcium Sulphate. Precipitated sulphur consists of masses of grouped spheroidal particles very similar to those of sublimed sulphur, but

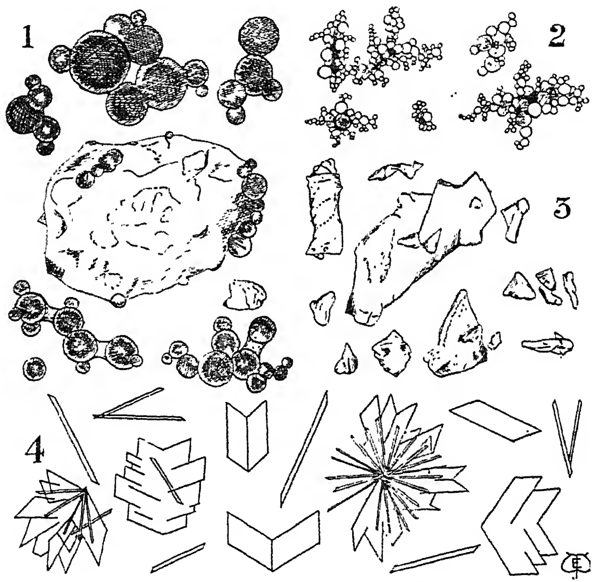

FIG. 32.—Commercial Varieties of Sulphur.

All × 200. (1) Sublimed sulphur. (2) Precipitated sulphur. (3) Powdered roll sulphur. (4) Crystals of calcium sulphate such as occur occasionally in samples of "milk of sulphur."

smaller in diameter, which varies from $1{\cdot}5\mu$ to 11μ; the single spheres appear nearly colourless, and may often show a tendency to angularity, see Fig. 32 (2). A variety of milk of sulphur that was formerly quite common contained a large proportion of calcium sulphate. One still occasionally meets with such samples, and the microscope will show the presence in them of colourless needle-shaped

crystals, and flattened twinned prisms similar to those shown in Fig. 32 (4) distributed among the sulphur particles. To exhibit these crystals it is best to moisten the specimen on the slide with alcohol and to mount in water.

When sulphur ointment is examined by warming with clove oil, as has been previously recommended for ointments generally (see p. 64), it will be found that both the lard and part of the sulphur dissolve in the oil, and on cooling crystals both of sulphur and of fat separate from the liquid, and have the appearance shown in Fig. 33.

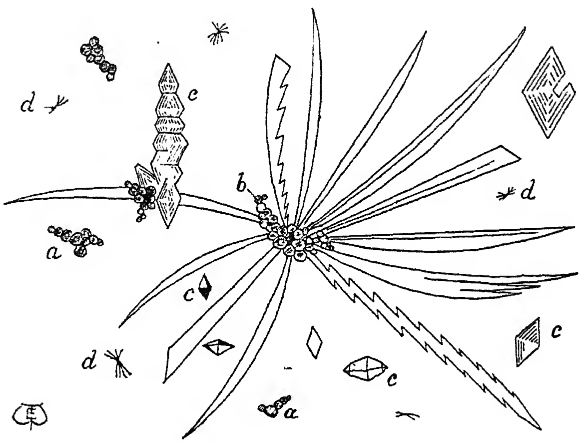

Fig. 33.—Sulphur Ointment after Treatment with warm Clove Oil.

(*a*) Unaltered sublimed sulphur. (*b*) Sublimed sulphur with sword-shaped sulphur crystals radiating from it. (*c*) Rhombic octahedra of sulphur. (*d*) Stearin crystals. All × 40.

The sulphur crystals are the usual characteristic rhombic octahedra, and to some extent in the form of sword-shaped crystals radiating from groups of sulphur spherules; the fat deposits small clusters of very transparent curved needles of stearin.

Precipitated Chalk. Precipitated chalk is another substance that should be subjected to a microscopical as

well as to a chemical examination when one wishes to form an opinion as to its suitability for any particular purpose. Its physical structure varies considerably in different samples, which are graded commercially as "extra light," "light," "medium," "heavy," etc. The chalk is used for pill-coating, for making tooth-powders, as a filling for "bible" and "cigarette" papers, to mix with salt to prevent caking, and for other purposes.

Precipitated chalk is generally characterized by stating its apparent density in pounds per cubic foot, which gives a certain indication of its physical condition, but does not tell one to what extent the powder is micro-crystalline or amorphous. As pointed out in the British Pharmaceutical Codex, the varieties that are preferred for tooth-powders are the more crystalline ones, and since the question of oral hygiene has come so largely into prominence in connection with dental practice, it is necessary to be prepared to discuss and explain the features which characterize the materials most suitable for the preparation of scientifically constituted dentifrices.

The examination of numerous commercial specimens of precipitated chalk shows that they frequently fail to comply with the "Characters and Tests" of the British Pharmacopœia, where this substance is described as a "white micro-crystalline powder." Some of the heavier varieties conform to this description, and the appearance of one of these is shown in Fig. 34 (1); the majority of the lighter powders, which are in great demand for certain purposes, show no distinctly crystalline particles, but have the appearance represented in Fig. 34 (2).

This "very light" powder consists of minute particles of no definite shape, and loosely adherent to form larger masses; this construction results in a maximum of air-spaces, and consequently such powders are specifically the lightest (compare the discussion on the "Texture of the Soil," in Chapter III of Hall's book, *The Soil*). Many "heavy" samples of precipitated chalk also show a lack of

crystalline structure, as shown in Fig. 34 (3). The individual particles are larger, and are aggregated into dense masses with no internal air-spaces, so that the powder is specifically heavier than those with a structure like that represented in Fig. 34 (2).

Since commercial precipitated chalks present so large a range of variation of structure, it is desirable to examine microscopically any sample which one intends to use for a particular purpose in order to see that it has a suitable physical constitution.

Prepared Chalk and Whiting. Prepared chalk is

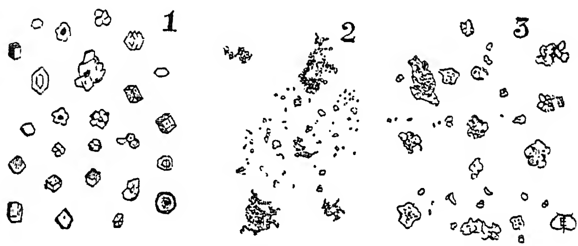

FIG. 34.—Commercial Samples of Precipitated Chalk.

(1) A "heavy" chalk showing a micro-crystalline structure. (2) A "very light" specimen, amorphous in structure. (3) A "heavy" chalk of a more commonly occurring type than that shown in (1). All × 200.

another chemical substance which has a very characteristic microscopical appearance. It is prepared from the native chalk found abundantly in various parts of the south of England. This chalk is powdered in a mortar and then mixed with water to form a cream, after which a large volume of water is added and the whole well stirred up. After standing for about fifteen seconds, the turbid liquid is decanted. The fine powder which settles from the decanted liquid is dried, and forms prepared chalk. Although it has been subjected to this rather vigorous treatment, one finds that the prepared chalk still contains many unbroken foraminifera, such as are shown in Fig. 27

(see page 81), while many of the finer particles are clearly recognizable as fragments of similar shells.

To examine a specimen of prepared chalk, a very small amount is placed on a slide, a drop of cresol is added, the two are stirred together, and a cover-glass is applied. The resulting preparation shows the structure of the microscopic shells very clearly.

Whiting, which is prepared by a similar process, may be examined by the same method, and exhibits a structure identical with that of prepared chalk.

CHAPTER IX

SUBLIMATION, PRECIPITATION AND STAINING

Sublimation. It is possible in certain cases to isolate from a complex mixture or a crude drug a definite substance in crystalline form by the method of sublimation, and, by using suitable apparatus, characteristic sublimates can be obtained from very small amounts of material. The microscopical appearance of these sublimates will frequently give valuable confirmatory evidence, or will suggest the course that should be followed in carrying out a systematic chemical investigation of the substance submitted for analysis.

Reinsch Test for Arsenic. One of the most reliable and most largely used of these tests is that known as the Reinsch test for arsenic. This is applicable to complex mixtures such as soup or viscera suspected to contain arsenic. In the case of viscera, a portion is put into a boiling-tube with some diluted arsenic-free hydrochloric acid (one volume of acid of sp. gr. 1·16 and about three or four volumes of water) and a spiral of clean copper foil, made by winding a narrow strip on a glass rod, is dropped in, and the whole boiled very gently for about half an hour. The organic matter becomes completely disintegrated, and any arsenic present appears as a dark deposit upon the copper spiral. When the boiling is finished the copper spiral is removed and washed by decantation with distilled water and with alcohol, and is then dried on a piece of filter paper in the steam oven. A piece of hard glass tubing, about 0·5 cm. in diameter, is now drawn off into a capillary tube at one end; the dried copper spiral is cut up

into small pieces and pushed into the other end of the tube. Holding the tube by the capillary, which should be open at the end, the copper clippings are shaken down to the constriction and the wider end of the tube is sealed up. By means of a Bunsen burner, heat is now gently applied until the arsenic volatilizes and becomes condensed in the capillary tube; this is then cut off and the sublimate is examined with the microscope. Well-formed octahedra are characteristic of arsenic. The freedom from arsenic of the materials used should be ascertained by boiling similar quantities with a copper spiral exactly as for the actual test, but with the omission of the suspected viscera, when no stain should be visible upon the copper. A positive control can be made by using a piece of ordinary meat in place of the viscera, adding a few parts per million of arsenic and proceeding as above; the sublimate obtained will serve for comparison with that from the suspected material, and will also enable some estimate to be formed as to the amount of arsenic present. The solubility of the crystals in reagents can be tested in the usual way. A drawing of crystals from a sublimate obtained by this test is shown in Fig. 35 (1).

Soup, tea, and other fluids are treated in a similar way, excepting that stronger acid should be added in such proportion as to yield a liquid containing about one-fifth of its volume of strong acid.

Microsublimation of Active Principles. A process of microsublimation is sometimes applied to crude drugs and other vegetable powders to obtain from them, in a condition exhibiting characteristic microscopical features, any volatile crystalline principles which they contain. The method is a development and extension of the test formerly used to distinguish genuine tea leaves from substitutes. Various methods of procedure have been advocated, and any which involve the direct application of a flame to watch-glasses or microscopic slides should be avoided, because these articles are very likely to be fractured and

the experiment lost. Tutin (*Pharm. Journ.*, Feb., 1912, 34, 157) has proposed a method of heating the drug in a small thin glass tube, sealed after the introduction of the powder and immersed in a bath of fusible metal, whose temperature is recorded by a thermometer. In this way a tolerably correct idea of the temperature of sublimation is obtained.

A further refinement has been proposed by R. Eder (*Schweiz. Woch. Chem. Pharm.*, 1913, 51, 228, and *Year-Book of Pharmacy*, 1913–4), who makes the sublimation at a reduced pressure, with the object of obtaining the sublimate in a purer and more definitely crystalline condition than when higher temperatures are used at the ordinary atmospheric pressure. The apparatus suggested provides for the registration of the temperature employed. A. W. Blyth (*Poisons: Their Effects and Detection*, p. 259) has also devised a mercury bath to be used with cover-glasses and a thermometer to conduct sublimations at controlled temperatures.

L. Rosenthaler (*Apoth. Zeit.*, 1913, 28, 991, and *Year-Book of Pharmacy*, 1914, 145) has suggested mixing the powdered drug with milk of lime, evaporating to dryness, finely powdering the residue and subliming it under reduced pressure in a suitable tube, when the sublimate can be examined microscopically or by any other method.

As pointed out by Tutin (*loc. cit.*) with reference to the microsublimation of scopoletin from gelsemium root, it is doubtful to what extent this method can be regarded as suitable or convenient for determining the presence and nature of the active principles in drugs and other powders, neither does it recommend itself as a reliable method for the detection of exhausted drugs in admixture with the genuine article. The method may, however, in certain instances, afford a valuable indication as to the best way of conducting an analysis by well-recognized chemical methods, or may serve as a useful sorting test for the

preliminary examination of a number of samples of the same substance.

For tea, gentian root, and other powders which readily yield a crystalline sublimate, the following method is useful:—A small crucible, or a shallow porcelain dish, not more than 1 in. (25 mm.) in diameter, is supported on a

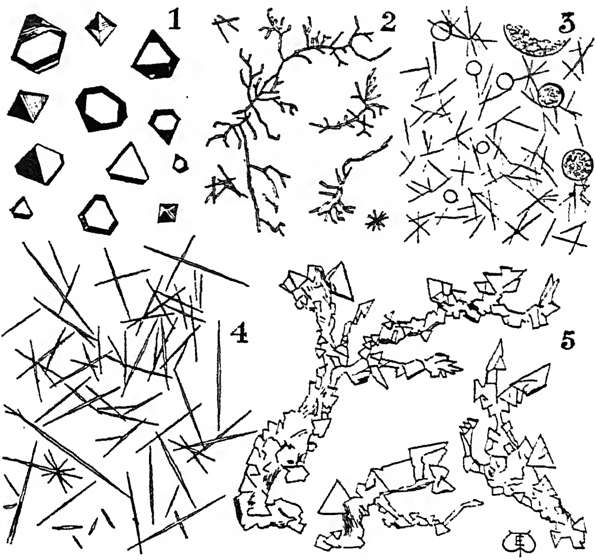

FIG. 35.—Various Microsublimates.

All × 200. (1) Crystals of Arsenious Oxide obtained by Reinsch's test. (2) Yellow crystals in the sublimate from rhubarb root. (3) Sublimate from gentian root with crystals of gentisin. (4) Caffeine Crystals from Tea. (5) Hydrastine Crystals from Hydrastis Rhizome.

sheet of asbestos cardboard from which a central hole has been cut to receive the bottom of the crucible or dish. A small amount—about 0·1 to 0·2 gm. of the drug—is placed in the crucible, which is covered by an ordinary microscope slide; heat is then gently and gradually applied, and the slide changed if much moisture or sublimate has collected. One should always have three or four clean

slides in readiness, when a sublimation is to be made. The process of condensation may be assisted by placing a small drop of cold water on the back of the slide. As the slides are removed from the crucible, put them aside until the sublimates have dried up, since the crystals frequently form slowly as the liquid present evaporates and the slide cools. They are then examined microscopically without applying a mounting medium or cover-glass.

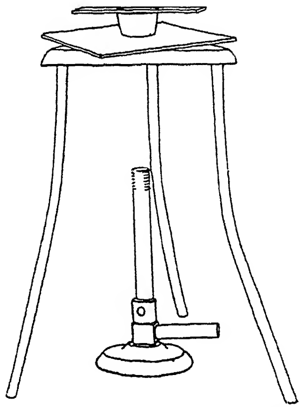

FIG. 36.—Apparatus for the Microsublimation of Crystalline principles from Drugs and other Materials.

A spirit-lamp may be used in place of the Bunsen burner.

Tea produces a sublimate of caffeine very easily. The sublimate is almost white if the heating is done carefully, and the crystals are in well-formed needles, as shown in Fig. 35 (4). Tea leaves that have been dried after having been used for the preparation of an infusion do not give a sublimate. Gentian root—Fig. 35 (3)—produces a yellowish sublimate, which has colourless circular or oval reticulate patches scattered throughout it, and small needles of gentisin separate from the yellow portion; these crystals cannot be obtained from exhausted gentian root. Hydrastis rhizome gives a yellow sublimate—Fig. 35 (5)—from

which very characteristic colourless crystals of the alkaloid hydrastine separate on cooling. Rhubarb root—Fig. 35 (2)—yields a sublimate from which small yellow crystals, frequently arranged in dendritic patterns, are deposited.

The appearance of the sublimates varies somewhat according to the rate of sublimation, amount of moisture, efficiency of cooling, and other factors, so that standard preparations from authentic materials should always be prepared at the time of experiment for purposes of comparison.

In connection with critical analytical work upon drugs, micro-sublimation has only a limited application (vide *supra*). For the detection of exhausted materials and for the isolation and identification of active principles one would prefer, in a general sense, to rely upon chemical methods. For the identification of vegetable drug adulterants in the form of powder, either substituted for or admixed with the genuine article, the histological characters afford the most reliable microscopical evidence.

Further details about sublimation methods will be found in *Pflanzenmikrochemie*, by O. Tunmann, in Chamot's *Elementary Chemical Microscopy*, and in the references quoted above.

Microchemical Precipitation. Much useful work has been carried out upon the precipitation of alkaloids and other substances, more particularly in connection with toxicological work, with a view to obtaining precipitates of characteristically formed microscopic crystals. The chief aim of this method of working is to enable the analyst to make a fairly comprehensive survey of the possibilities when the amount of available material is strictly limited. There are also a few instances where the microscopical structure of the precipitate is the most reliable method of distinguishing between two or more closely allied substances, as in the case of lactose and maltose, whose osazones have a characteristic microcrystalline structure of diagnostic value.

Osazones. To prepare osazones from sugar solutions, one proceeds as follows: Into a clean test-tube put 0·1 gm. of phenylhydrazine hydrochloride and 0·3 gm. of sodium acetate and add about 5 c.c. of the sugar solution. Heat the mixture by standing the tubes in the water of a boiling water-bath for thirty to sixty minutes. On removing the tubes allow them to cool slowly and yellow crystals of the osazone separate. A small quantity of the crystals is removed from the test-tube in a dipping tube and mounted for microscopic observation. Drawings of the osazones of glucose, lactose and maltose are shown in Fig. 37.

Phenyl-glucosazone consists of fairly large straight needles arranged in feathery groups; phenyl-lactosazone crystallizes in rosettes of very fine hair-like crystals, many of which are strongly curved; phenyl-maltosazone forms rosettes of thin, broad, flat crystals usually with blunt or rounded ends.

Precipitation Tests. In making precipitation tests upon the slide, there are various ways of applying the reagents. A good plan is to use the modified irrigation method, or one may place the two drops of reagent and liquid to be tested side by side upon the slide, and observe their coalescence under the microscope. In many cases it suffices to mix the two drops placed closely together upon the slide by lowering on to them a cover-glass, and then watching through the microscope for the appearance of a precipitate, which in most cases will form gradually if the solutions used are, as they should be, quite dilute. Another very convenient method is to mix the drops in a small watch-glass, and observe the effect with the microscope. In the case of a volatile substance, a drop or two of the liquid may be placed in a watch-glass or dish and a microscope slide, with a drop of the test liquid suspended from it, placed across the rim; after a sufficiently long exposure to the vapour the slide is quickly inverted, and the drop examined with the microscope, either with or without the application of a cover-glass. When working with alkaloids,

the strongest solutions that need be employed will be about 1 per cent., and the reagents most largely used are ammonia (one volume of solution of ammonia sp. gr. 0·880 diluted to ten volumes with water), potassium or ammonium

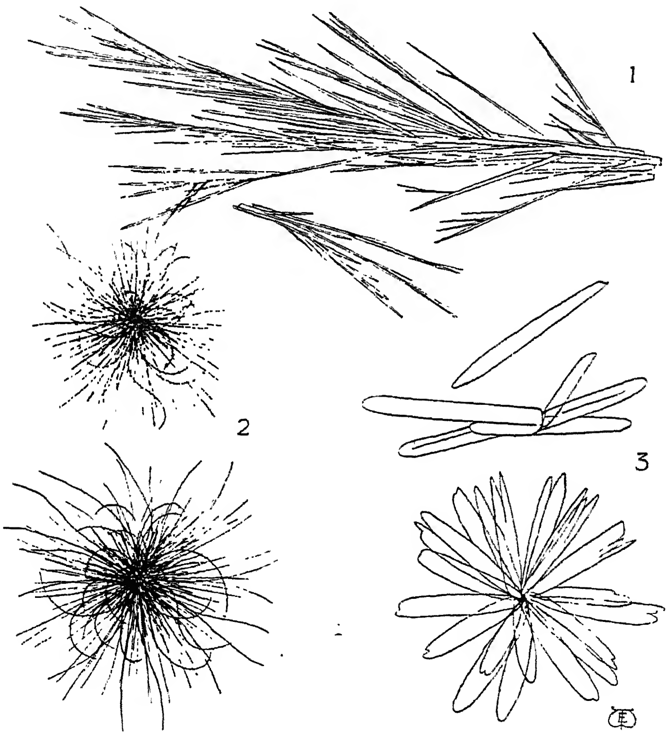

FIG. 37.—Osazones of Various Sugars. All × 300.
(1) Phenyl-glucosazone. (2) Phenyl-lactosazone. (3) Phenyl-maltosazone.

thiocyanate in 5 per cent. solution and picric acid. Most of the ordinary tests can be carried out upon the microscope stage, if it is desirable to do so. In all cases one should compare the results obtained with those yielded by the interaction of pure materials in approximately

the same proportions and strength of solution as used for the actual test.

Good drawings of the precipitates from various poisonous inorganic and alkaloidal solutions are helpful to the analyst in coming to a decision as to the nature of the substance present, but conclusions formed by comparison with such drawings should always be confirmed by carrying out control tests as suggested above. Excellent drawings of a number of important microchemical precipitates are to be found in T. G. Wormley's *Microchemistry of Poisons.* Photographs of a large number of alkaloidal precipitates are to be found in *Some Microchemical Tests for Alkaloids*, by C. H. Stephenson. Further information about microchemical testing in general may be found in H. Behrens' *Anleitung zur Mikrochemischen Analyse.*

Stains and Staining. There are very few stains that are useful in analytical work. The two most serviceable ones are corallin and ruthenium red. Corallin is used dissolved in a 25 per cent. solution of crystalline sodium carbonate. The corallin is added to the sodium carbonate solution until a bright pink colour is produced. The reagent should be freshly prepared.

Corallin stains red the callus plates of sieve tubes, and for this reason is very useful to aid in the identification of a powder as one that contains phlœm tissues—generally a bark. It is, in fact, one of the most important positive tests for the recognition of powdered barks. The sieve-plates in material that has been disintegrated after digestion with caustic soda or potash are stained red by corallin-soda quite as well as sieve-plates in untreated material or such as has been bleached by chlorinated soda.

Ruthenium red is used as a stain for mucilage. The solution is prepared by adding ruthenium red to a 10 per cent. aqueous solution of lead acetate until a wine-red colour is produced. The reagent should be freshly prepared. One of the most important uses of this stain is for the recognition of cacao shell added to cocoa powders.

The only part of the cacao seed which contains mucilage is the seed-coat, and this should be removed during the manufacture of powdered cocoa and of chocolate. Hence the addition of powdered cacao shells to cocoa can be detected by staining the mucilage with ruthenium red. A count of the number of stained particles present in the powder has been used as an approximate method of quantitative estimation of the amount of shell present.

Staining Sections. The necessity for making sections and preparing permanent mounts arises occasionally in the course of analytical practice, especially when some new feature has been detected and the worker wishes to communicate the result to his fellows. There are many different ways of staining and mounting sections of vegetable tissues, but only one need be considered here. The most permanent mounts are those made by the use of Canada balsam as a mounting medium, and it is generally desirable to use two stains, one of which colours the cellulose tissues, while the other colours those that are lignified. Such a method of staining is termed double staining, and a very good solution is hæmatoxylin, which stains cellulose blue, and safranin, which gives a bright red colour to the woody elements.

Hæmatoxylin and Safranin. The best hæmatoxylin stain for this purpose is that known as Delafield's, the formula for which is thus given by Squire in his *Methods and Formulæ* :—" To 400 c.c. of a saturated aqueous solution of ammonia alum add 4 gms. of hæmatoxylin dissolved in 25 c.c. of absolute alcohol; leave the solution exposed to the light and air in an unstoppered bottle for three or four days; filter, and add to the filtrate 100 c.c. of glycerin and 100 c.c. of methylic alcohol (wood spirit); allow the solution to stand in the light until it is a dark colour, re-filter, and preserve in a stoppered bottle. (Ammonia alum dissolves about 1 in 11 of water.)"

The safranin solution is a saturated solution of safranin in 70 to 90 per cent. alcohol.

The most rapid and satisfactory way of preparing a section is to carry out the whole operation upon the microscope slide, and the method of procedure is as follows :—

Make sections with a razor flooded with alcohol ; transfer to a slide, and, if necessary, add a drop of chloral hydrate solution to clear the preparation. Remove the chloral by tilting the slide and allowing as much as possible to drain off, and then wash by adding successive drops of water and draining off ; finally wash similarly with alcohol of the same strength as that used in the preparation of the hæmatoxylin solution. About 70 per cent. alcohol is generally suitable. Drain off the alcohol and add a drop of Delafield's Hæmatoxylin Solution, and allow to stand for a few minutes till sufficiently stained, which can best be judged by draining off, washing with a few drops of dilute alcohol, and examining with the microscope. Next wash with distilled water, and finally with hard tap-water (or a very dilute aqueous solution of sodium bicarbonate, about 1 in 1000). Wash again with distilled water, followed by dilute alcohol, and finally stronger alcohol of the same strength as that used for the solution of safranin. Drain off the alcohol, and add a drop or two of safranin solution and allow to stand for five minutes or so, being careful not to allow the section to become dry ; then drain off the excess of safranin and wash with alcohol until the washings drain from the slide practically colourless, the final washing being made with the strongest alcohol (97 to 100 per cent.). Now drain off the alcohol and add a drop of clove oil, and examine with the microscope to see that the section is properly cleared. Remove the clove oil, by draining it off and wiping away the residue with a piece of ordinary paper, or transfer the section on the point of a needle to another slide, and add a drop of xylol-balsam and apply a cover-glass. Benzol- or xylol-balsam is prepared by dissolving the hard resin of Canada balsam in benzol or xylol to form a solution having the consistence of a thin syrup. The hard resin is obtained from the crude Canada balsam by

heating it in an open dish on a water-bath until the volatile oil has been driven off and the residue sets, on cooling, to a hard brittle mass.

When mounting delicate structures or sections of very young tissues, there is sometimes a little difficulty in transferring the preparations from the alcohol to clove oil, which may cause shrinkage and spoil the sections. The difficulty may be surmounted by passing them first into a solution of clove oil in alcohol, and finally into clove oil, or (as suggested by W. Mark Webb in his book, *The Microscope*) some clove oil may be poured into a small flat-bottomed tube to a depth of about ½ in., and some alcohol carefully poured on to the top of it, so that no mixing takes place. The section is then dropped into the alcohol, through which it sinks to the level of the oil, which gradually penetrates the tissues and the section eventually falls to the bottom of the tube. The alcohol is then removed by a pipette, and the cleared section is taken out and mounted in xylol-balsam. If this method is unsuccessful, the section can be taken from alcohol to water and mounted in glycerin jelly.

Mounting in Glycerin Jelly. For this purpose a drop of glycerin jelly is placed on a microscope slide and allowed to set; the wet section is placed upon the jelly, and the slide is gently warmed, when the section sinks through the jelly, which is then covered by a cover-glass. If this is done carefully no bubbles should appear. Further particulars will be found in a paper by L. W. Stansell in the *Analyst*, September, 1913, p. 407.

The following is a good formula for glycerin jelly :—

Gelatin	20 gms.
Distilled water	150 c.c.
Glycerin	100 c.c.
Phenol	2 gms.

Soak the gelatin for two or three hours in sufficient of the water to cover it, and then dissolve by warming on a water bath. Dissolve the phenol in the glycerin and the remain-

der of the water, and add to the gelatin solution. Mix and filter through paper while warm, using a jacketed funnel.

This formula yields a glycerin jelly suitable for all ordinary purposes. If, however, an absolutely clear jelly is required, it is necessary to clear the gelatin solution before adding the phenol. This is done by cooling it to 60° C., mixing in the well-beaten white of an egg, and boiling for a few minutes to coagulate the albumen. The mixture is filtered through a filter paper in a hot-jacketed funnel, and to the clarified liquid an equal volume of a warm 2 per cent. solution of phenol in glycerin is added.

CHAPTER X

MEASUREMENT AND DRAWING

Drawing and measurement necessarily go together, for, in most instances, a drawing made for identification purposes —and it is for that reason that the practitioner needs his drawings—must either be executed to scale or must have the dimensions marked against it. In some cases a knowledge of the exact size is not necessary for identification; but, even under these circumstances, comparison with published drawings and descriptions is greatly facilitated, and confidence in one's conclusions is increased by knowing the size of the object.

In the case of drawings intended for publication, they must always be made to scale, and the magnification must be stated in diameters, or a scale of microns added to the drawing, as shown in the accompanying drawing of crude fibre and (below the line) cell contents of Black Pepper.

Micrometers. Two scales are needed for making microscopic measurements. One of them, termed a stage micrometer, takes the form of an ordinary mounted microscopic object, and consists of a scale 1·1 mm. long, 1 mm. being divided into tenths and the odd tenth divided into hundredths. This scale is engraved or photographed upon an ordinary microscope slide, and mounted under a cover-glass like any other object. It is intended to be placed upon the stage of the microscope, and is viewed through the instrument.

The second scale, named an eyepiece micrometer, consists of a small disc of glass to be dropped on to the diaphragm of an Huyghenian eyepiece. Across the centre of the disc is

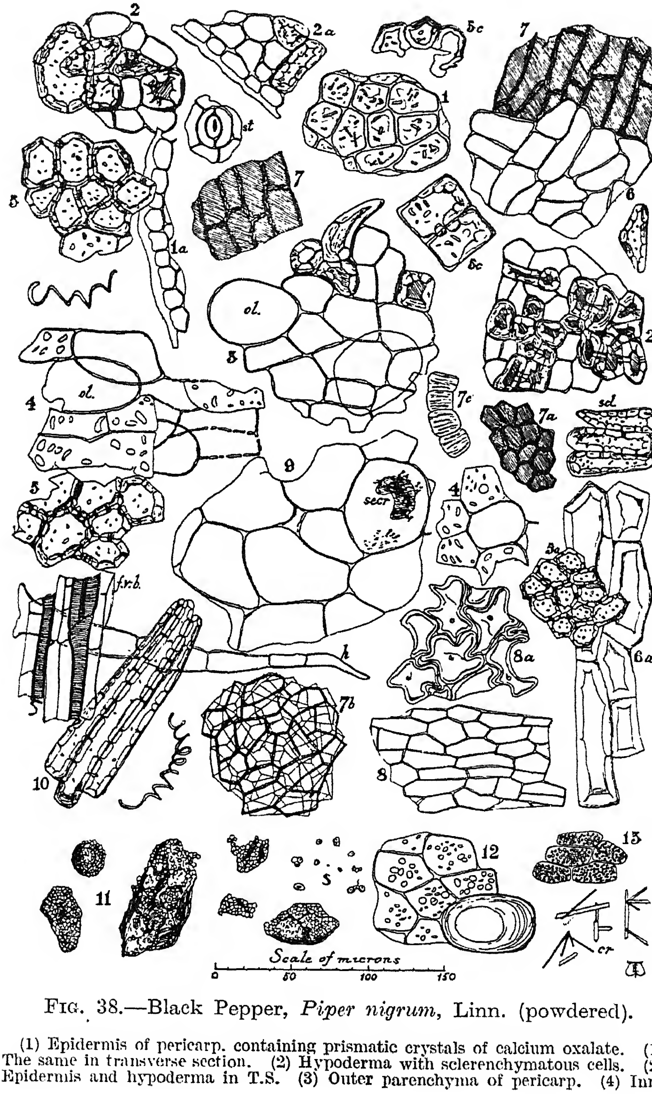

FIG. 38.—Black Pepper, *Piper nigrum*, Linn. (powdered).

(1) Epidermis of pericarp. containing prismatic crystals of calcium oxalate. (1*a*) The same in transverse section. (2) Hypoderma with sclerenchymatous cells. (2*a*) Epidermis and hypoderma in T.S. (3) Outer parenchyma of pericarp. (4) Inner

engraved or photographed a scale divided into units and tenths of units. The exact size of the unit chosen is immaterial, but it is customary to make the scale 10 mm. long, each millimetre being divided into tenths.

A second form of eyepiece micrometer is needed for counting particles under the microscope, and is also very useful for drawing large objects without a camera lucida. This is a squared or net micrometer, consisting of a circular disc of glass having a central area of 1 sq. cm. ruled into 100 small squares, each 1 sq. mm. in area.

It is most convenient to set aside a special eyepiece for the micrometer scale, which is allowed to remain permanently in position upon the diaphragm in the middle of the eyepiece tube. If the scale is not properly in focus, this should be rectified by readjusting the eye lens. When one wishes to make a measurement, this eyepiece is exchanged for that ordinarily in use.

Camera Lucida. Another way of making measurements is by means of a camera lucida, such as is used for drawing. There are many types of this instrument, and the better ones are those which permit of the microscope being used in a vertical position. The Abbé model is a very good pattern, but is rather heavy, and hence liable to strain the microscope. The most convenient type of camera lucida is the Swift-Ives pattern, which is quite a small instrument, and may be attached to the cap of a capped eyepiece, as shown in the figure (Fig. 39), or can be adapted to fit over the upper or eye lens of an ordinary eyepiece, and is then clamped in position by a small screw.

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parenchyma of pericarp. (5) Inner sclerenchyma of pericarp. (5*a*) The same from the apex of the fruit. (5*c*) The same seen in profile. (6) Innermost layer of the pericarp. (6*a*) The same from the apex of the fruit. (7) Pigment layer. (7*a*) The same from the apex of the fruit. (7*b*) The same from the base of the fruit. (7*c*) Pigment layer from apex of the fruit seen in profile. (8) The hyaline layer. (8*a*) The same from the apex of the fruit. (9) Cells of the perisperm. (10) Sclerenchymatous cells accompanying the fibro-vascular bundles. (11) Masses of starch from the perisperm. (12) Outer parenchyma of the pericarp showing starch grains and an oil cell. (13) Cells with aleurone grains from outer part of the perisperm. *h*, hair from bracts under the fruits. *f.v.b.* Portion of fibro-vascular bundle. *s*, Isolated starch grains. *scl*, sclerenchyma of hypoderma of pericarp. *st*, Stoma from epidermis of pericarp. *ol*, Oil cell. *cr*, Crystals of piperin obtained by treating the powder with alcohol followed by dilute glycerin. *scr*, Remains of oily contents of oil cell in the perisperm. All × 200.

With this instrument, the paper, lying upon the table or upon a drawing-board beside the microscope, is reflected by the small movable right-angled prism A into a second prism, B, above the eye lens E of the ocular. The face CD of this prism is silvered, and reflects the light vertically upwards into the observer's eye, while the microscope image is viewed directly through a small central opening in the silvered surface (see dotted line in the figure). As a result of this optical arrangement, the image in the micro-

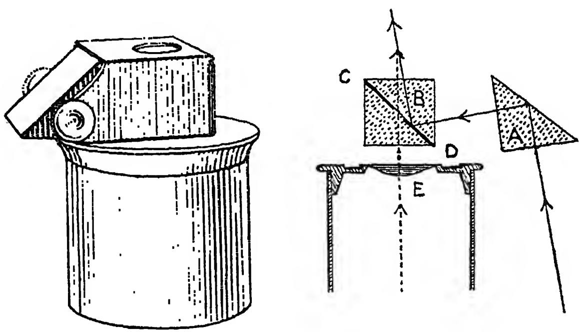

FIG. 39.—The Swift-Ives Camera Lucida and a diagram to explain the Path of the Light Rays through the Instrument. (See text.)

scope and the paper are seen superposed one upon the other, while a pencil used on the paper also comes into the field of view, so that one can easily trace over the image seen in the microscope.

The whole instrument is quite small, and measures about 35 × 17 × 18 mm.; the total weight, including the eyepiece cap, is less than 50 gms. (1·75 oz.).

One should, if possible, keep an eyepiece specially for use with the camera lucida; such an eyepiece should have a low magnifying power of about six diameters.

Micrometry. Before a measurement can be made, the value of the divisions of the eyepiece micrometer must

be determined. This is accomplished by placing the stage micrometer upon the stage of the microscope and focusing the lines engraved upon it, using for the purpose the objective with which the measurements are to be made and the ocular containing the eyepiece micrometer. The appearance of the field of view will resemble that shown in Fig. 40.

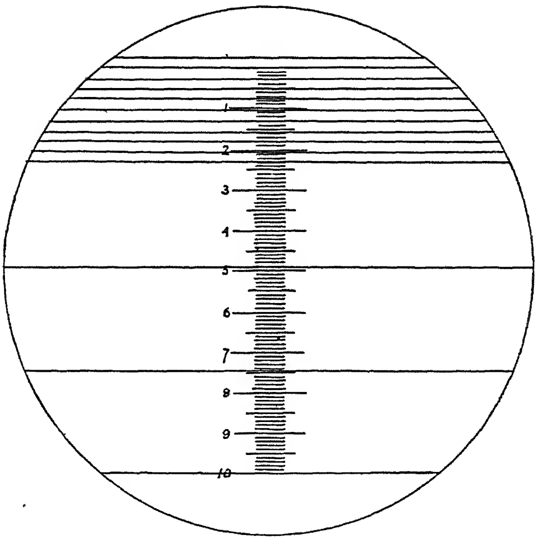

FIG. 40.—Appearance of Eyepiece and Stage Micrometer Scales as seen through the Microscope with a 4 mm. Objective and a × 6 Eyepiece.

100 Eyepiece scale divisions (marked 1 to 10) are seen to be exactly equal to 3·9 of the stage micrometer divisions, *i.e.*, to 0·39 mm.

In this figure, the lines reaching quite across the field represent the image of the rulings upon the stage micrometer, while the shorter lines of the numbered scale represent the rulings of the eyepiece micrometer. If there should be no exact coincidence between the extremities of the eyepiece scale and two of the rulings of the stage micro-

meter, the draw-tube of the microscope must be extended until coincidence occurs. One now determines the number of stage micrometer divisions which are exactly equivalent to the hundred divisions of the eyepiece scale; in the figure this number is 3·9 divisions. Since the rulings of the stage micrometer are 0·1 mm. and 0·01 mm. apart respectively, we have the result that 100 eyepiece divisions are equivalent to 0·39 mm., or each division of the eyepiece scale represents, with this particular optical combination, 0·0039 mm. or 3·9μ (one micron—μ—is 0·001 mm.).

If now the stage micrometer is removed and any ordinary object is viewed through the same lenses, without altering the tube-length, one can read off its dimensions in terms of the divisions of the eyepiece scale, and these numbers multiplied by 3·9 give the actual dimensions in microns.

When using a camera lucida, measurements can be made either with a stage micrometer or a calibrated eyepiece micrometer. In either case the scale is traced off upon the drawing-paper and, the actual value of these divisions being known, this immediately gives the scale by which the dimensions of the object can be obtained from the drawing made upon the paper by means of the camera lucida.

Identification by Measurements. Measurements are frequently necessary for the qualitative identification of closely allied substances. For example, Rio or Brazilian Ipecacuanha root, obtained from *Psychotria Ipecacuanha*, Stokes, contains starch grains which never exceed 15 microns in diameter and is thus distinguished from Carthagena Ipecacuanha, which is attributed to *Psychotria acuminata*, Karsten, and has starch grains, many of which measure between 17 and 22 microns. In a similar way Cassia Bark, from *Cinnamomum Cassia*, Blume, has starch grains measuring from 10 to 20 microns, while cinnamon bark, from *Cinnamomum zeylanicum*, Breyn, has smaller starch grains, usually from 6 to 8 microns.

Wheat starch, also, can be definitely distinguished from barley starch only by measurement of the largest grains

it contains. Barley starch grains never exceed 40μ in diameter, while the largest wheat starch grains attain a maximum diameter of about 50μ.

Measurement of the size of red blood corpuscles will aid the analyst in forming an opinion as to the origin of the blood. The red corpuscles of human blood measure 7·25 to 8μ with an average of $7{\cdot}5\mu$, while those of many other mammals are smaller, *e.g.* dog $7{\cdot}3\mu$, rabbit $6{\cdot}9\mu$, rat $6{\cdot}7\mu$, cat $6{\cdot}5\mu$, ox $6{\cdot}0\mu$, horse $5{\cdot}4\mu$, sheep $5{\cdot}0\mu$, goat $4{\cdot}0\mu$. Red blood corpuscles of the Mammalia have no nucleus, but those of birds, amphibia, reptiles and fishes are all nucleated and, with few exceptions, are oval in shape.

Value of Drawing. Drawing is of the greatest value in connection with microscopy. It is a kind of universal written language, and enables one to express many facts that it is impossible to describe in words. Even when words are adequate for descriptive purposes, a clearer impression, and one that is much more quickly grasped, is often conveyed by means of drawings used either alone or as supplementary to what is written.

No elaborately finished drawings are needed; all that is required is an accurate outline shape, such as can be produced by anyone who will spend a little effort in practising with the microscope.

It will be found a good plan to shade the eyes, when possible, from any direct light, and the small metal shade suggested as an attachment to the chimney of an oil-lamp, as shown in Fig. 3 (see p. 8), gives great relief to the eyes. Similarly, it is good to lower a dark blind sufficiently to shade the face, if one is working in front of a window with a strong light from the sky.

If the microscopist is able to do so, it is most comfortable to use the left eye for looking through the microscope, while the paper and pencil are placed on the right-hand side of the instrument. It is then possible to keep the image much more closely under observation while making the drawing than is the case if the right eye is used.

All drawings should be made to a scale sufficiently large to enable the worker to introduce all necessary details. As a general rule, it will be found advisable to make sketches about twice the apparent size of the image seen in the microscope. The magnification obtained with a $\frac{1}{6}$ in. (4 mm.) objective is about 300 diameters, so that, with this objective, the drawings should be made to a magnification of about 600 if one wishes to include minute details. Similarly, for a $\frac{2}{3}$ or $\frac{3}{4}$ in. (17 or 19 mm.) objective, a magnification of about 300 is suitable.

Freehand Drawing. The making of rapid freehand sketches is the most useful kind of drawing for regular use in the laboratory. The eyepiece should contain a calibrated scale, and one should use a soft (B) lead pencil or a smoothly working pen. Any ordinary paper with a fairly good surface will suffice for such work.

This method of drawing is particularly useful for the identification of the constituents of water deposits. Many of the objects are in motion, and one needs to practise taking measurements rapidly and making swift sketches to show the important features. The measurements are most conveniently recorded against the sketches in terms of scale divisions, being converted into microns later on when comparing the sketches with reference drawings. The accompanying figure (Fig. 41) shows how one may quickly record the general appearance and the measurements of a miscellaneous collection of objects such as occur in a water deposit.

In the same way one should jot down sketches of any particular features found in powders and other substances while under examination. This method creates a satisfactory record of the salient points, and saves the worker from a feeling of uncertainty when comparing his observations with published drawings or with authentic material.

Drawing to Scale. All drawings intended for publication or for use as permanent records must be made to scale. There are various methods of doing this, and those

depending upon the use of scales in the eyepiece are applicable under all circumstances and to every kind of object; an acquaintance with these methods is therefore essential.

Drawing with a camera lucida is not always possible, owing to difficulties of illumination or to variation in the quality of the preparations. In the majority of cases, however, the camera lucida can be employed; and, if one has much accurate drawing to do, this instrument should be used whenever possible in order to economise time.

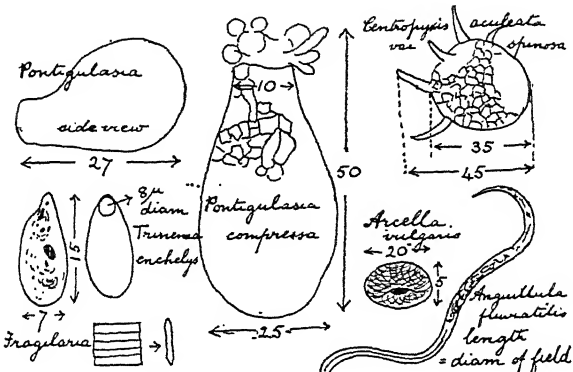

FIG. 41.—Rough Sketch showing how Freehand Notes may be made of Objects in a Water Deposit.

The names are added after comparison with standard figures and descriptions. The figures represent dimensions in eyepiece scale divisions using a 4 mm. objective. Each division was equal to 2·8 microns. The diameter of the field was 0·36 mm.

Use of Eyepiece Scales. The ordinary graduated eyepiece micrometer scale, described above, is sufficient for making accurate drawings. In this case one measures the size of the various structures present, and, having multiplied by the magnification desired, one sets out the proportions on the drawing-paper, and the sketch is finished freehand.

This may appear to be a tedious process, and if carried out exactly as stated, it is so. The difficulties are greatly

minimised by constructing a set of scales for use in this connection.

First of all make scales representing microns at definite magnifications such as are being constantly used for the drawings. These magnifications should be simple round numbers, all odd figures being carefully avoided. Useful figures are 400, 200, 100, 80, 50, and 40 diameters, and two sets of figures can be represented on the same scale, one above and the other below the line, as shown in Fig. 42.

The scales when constructed are not only useful for making drawings, but also serve for finding the sizes of objects from reference drawings made at the same magnifications.

It is well to make a habit of using the same magnification for all drawings made with the same combination of lenses; all one's figures will then be comparable with one another, and a correct idea of relative size of different details is obtained by looking from one drawing to another.

A second series of scales should now be made showing the divisions of the eyepiece scale at the various magnifications as seen with any particular combination of lenses. For example, one could make scales showing the eyepiece micrometer scale at 400 and 200 diameters with the micrometer ocular and a $\frac{1}{6}$ in. (4 mm.) objective; at 100, 80, and 50 diameters with the $\frac{2}{3}$ (17 mm.) objective, and at 40 and 20 diameters with the $1\frac{1}{2}$ in. lens. The scales are constructed by determining the value of the eyepiece divisions in the usual way, and, by using the scales of microns already prepared, marking off the divisions at the required magnification. Finally, write against each scale the magnification and the lens combination used.

For example, in constructing the scales representing eyepiece divisions in Fig. 42: With the 4 mm. objective and a $\times$ 6 eyepiece, one eyepiece scale division was equal to 3·9 microns, hence 50 divisions equalled 195 microns. To represent the scale at $\times$ 200, 195 microns is measured off on the first scale of microns, A in Fig. 42, and marked upon

the scale H, where it is numbered 5, as upon the actual micrometer scale. This length is then divided, by the well-known geometrical construction, into five equal parts, and the first part is further subdivided into tenths. The other scales, G, K, and M are similarly constructed. It is a good plan to draw all the scales upon the same piece of Bristol board as shown in the figure.

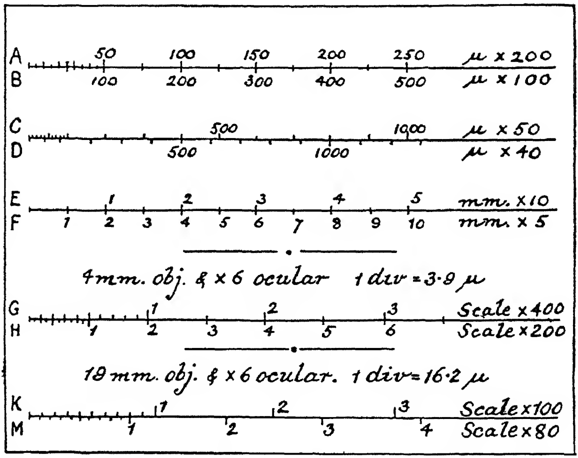

FIG. 42.—Scales for Use in Making and Using Drawings.

The scales of microns and millimetres (A to F) are those corresponding to the illustrations in this book. The scales (G to M) are those required for making the drawings by the use of an ordinary eyepiece micrometer scale. (See text.)

To draw at × 200, using the 4 mm. objective and × 6 eyepiece, one observes the dimensions of the structures in terms of the eyepiece micrometer scale divisions, and from the scale H of Fig. 42, one measures off immediately the dimensions which will represent those structures correctly at the required magnifications of 200 diameters.

Use of the Squared Micrometer. An eyepiece micrometer having a central area of one square centimetre

divided by fine lines into 100 small squares, each one sq. mm. in area, is most useful for drawing objects which fill the field of view in the microscope, and more particularly for use with low-power objectives, in sketching such things as legs, heads, and other parts of insects, and making diagrammatic drawings of sections to show the distribution of the tissues.

The image in the microscope is seen covered by the network of lines, and the first thing to be done is to draw to scale a similar set of lines over the whole of the sheet of drawing-paper. For this purpose one determines the value of the eyepiece micrometer divisions, and then constructs a scale to represent them at the desired magnification as described above. The scale is marked off along the margins of the paper and the squares are ruled in. The image is then drawn over the squares in much the same way as one draws the outline of a map by using the parallels of longitude and latitude. An example is given in Fig. 43. In this way one gets the main features in their correct relative positions. Measurement of individual details, when needed, must be made in the usual way with an ordinary eyepiece scale, and are filled into the drawing by the method described above. The finished drawing is lined in with ink, and the pencilled squares are removed with india-rubber.

Drawing with a Camera Lucida. When drawing with the camera lucida, the illumination of the object and paper must be carefully balanced, so that the pencil and image are seen equally well in the microscope tube. The next point to attend to is the magnification, which should be adjusted to some round number like 200 or 100, etc., as previously suggested. This is arranged by using the stage micrometer as the object to be drawn, and adjusting the tube length of the microscope, or the height of the paper from the table, or both, until the distance between the tracings of the micrometer lines as seen on the paper gives the exact magnification desired. This is a very simple

matter to adjust in practice, and, having done so, all drawings made on the same paper will be at that particular magnification.

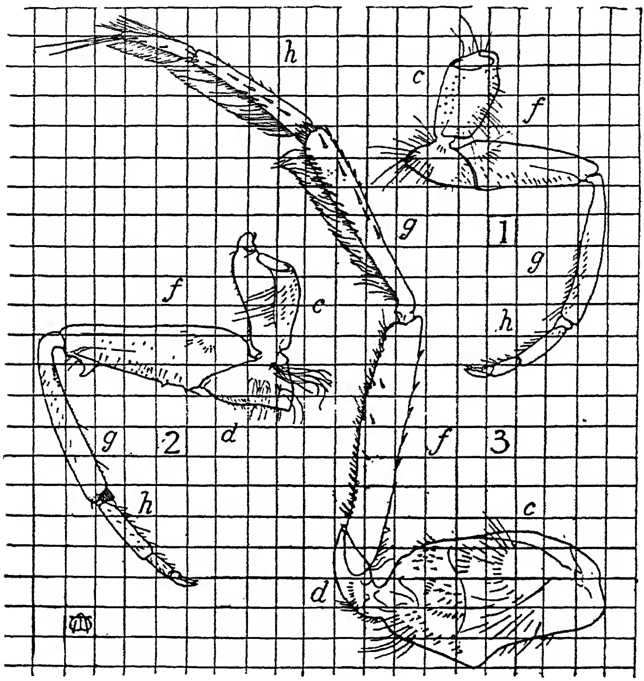

FIG. 43.—Drawing, made with a Squared Micrometer, of the legs of the Water-Boatman, *Notonecta undulata*, Say (Mexican Cantharides).

(1), (2) and (3), fore, middle, and hind legs respectively, *c*, coxa; *d*, trochanter; *f*, femur; *g*, tibia; *h*, first joint of the tarsus. All × 10. The squares have been left on the drawing to show how it was made.

After adjusting the length of the draw-tube, it may be prevented from slipping by fixing upon it a small metal ring fastened by means of a thumb-screw. Such a ring will be made by any microscope manufacturer.

The camera lucida does not help one to put in all the fine details of a drawing. Its only use is to mark the main outlines and the position of the principal features so that the general proportions are correctly expressed. All the fine detail must be filled in by freehand afterwards.

Drawing Materials. When making drawings for reproduction, one should select a fairly substantial paper with a good, smooth surface. A suitable paper is Hollingworth's " Improved " Drawing Paper, " hot-pressed," and a convenient size is " Royal."

The pencil used will vary a little according to individual fancy, since one can produce much the same effect with a softer pencil, used lightly, as with a harder one used more heavily; generally speaking, a moderately hard pencil (H or HB) is best for careful work.

Lining-in must be done with a good, black Indian ink, such as Reeve's " Fixed Indian Ink." The pen nibs must be carefully chosen, and, while any good drawing-pen is suitable for ordinary use, for the finest work a better pen, such as Joseph Gillott's Lithographic Pen No. 290, should be used.

Drawings and Photographs. One must guard against the assumption that published drawings show all the structures and other features exhibited by any given material. Just as a written description may be faulty or partial, so also drawings, owing to inaccurate observation, may not represent all the structures present. One is, therefore, well advised, when unable to find in the reference drawing what one sees with the microscope, to make a comparison with preparations of authentic material, before passing judgment upon the specimen under examination.

From this point of view, photographs are no more satisfactory than drawings, because only one field can be shown in the photograph, and it is seldom that all the characteristic elements of a substance are to be found in a

single field. The difficulties of focusing are also so great that details from all parts of the field are rarely equally well represented in the photographic print.

In the case of a drawing, one selects the useful features from a number of fields and preparations, and this results in a presentation in one drawing of all the salient facts so far as the worker has been able to observe them. Drawings also show an advantage in that important features can be emphasised and indefinite blurred patches need not be introduced unless they have diagnostic value.

CHAPTER XI

QUANTITATIVE MICROSCOPY

Aim of Quantitative Work. The aim of quantitative microscopical work is to make measurements of mass. Such work is still in its initial stage, and can only be extended as further research clears away the many difficulties and uncertainties which surround most of the problems to be solved.

Since the microscope is merely an aid to vision, it is obvious that weighings cannot be made directly; they must be estimated by counting the number of particles of a particular kind or by measuring the superficial area of portions of materials having a definite thickness and a known density. Both methods are used.

Method Based on Measurements of Area. One of the oldest methods was applied by petrologists to the determination of the proportions of the various minerals present in rocks. For this purpose one makes an exact drawing to scale of the section of rock, preferably by the aid of a camera lucida. The areas of the various pieces of mineral are then measured by a planimeter or by some other well-known method, and the volumes calculated from the known thickness of the section. From the density and the volume one obtains the mass of the various constituents present, and the percentage proportions can be immediately calculated. Instead of making a drawing, one may photograph the specimen with a net-ruled micrometer in the eyepiece. The value of the micrometer squares is ascertained and the areas of the component minerals are calculated from the photograph.

The method is based upon the work of A. Délesse upon the constituents of rocks (*Comptes Rendus*, 1847), and has been successfully used by Sollas (*Trans. Roy. Irish Acad.*, 1887–1892) for the examination of granites, by J. Joly (*Trans. Roy. Irish Acad.*, 1903–5) for paving sets, and by Johnson (*Eng. Record*, 1915) for cements and concretes.

Counting Methods. Counting methods are those which are most generally serviceable. Their accuracy depends chiefly upon the uniformity of the particles to be counted and the evenness of their distribution through the material under examination.

The method used for making such counts should be one that requires no special apparatus, and which can be carried out equally well on any microscope and with any combination of lenses which gives a suitable magnification. The only method, hitherto suggested, which fulfils these conditions is the Lycopodium method, worked out by Wallis (*Analyst*, 1916, pp. 357–374).

Function of Lycopodium. The lycopodium is added to the substance under examination in a definite proportion by weight, and its function is to enable one to know in what weight of material the characteristic particles have been counted.

Lycopodium is composed of the spores of *Lycopodium clavatum*, L. Each spore is tetrahedral in shape, the base is rounded and the three flat sides meet to form three well-marked converging ridges, which join one another at the apex. The whole surface of the spore is covered with minute reticulations and the interior is filled with fixed oil. These details are shown in Fig. 44.

Lycopodium spores are exceptionally uniform in size, so that one can always know that a definite number of spores represents a particular weight of lycopodium. The whole process is simplified by knowing the weight of a single lycopodium spore, or, as it is more conveniently expressed, the number of spores per milligramme. This quantity has been determined (Wallis, *Pharm. Journ.*, July, 1919,

p. 75), and shown, as the result of a large number of experiments, to average 94,000 spores per milligramme.

Using this figure one can calculate the weight of any number of spores counted under the microscope, and, if the

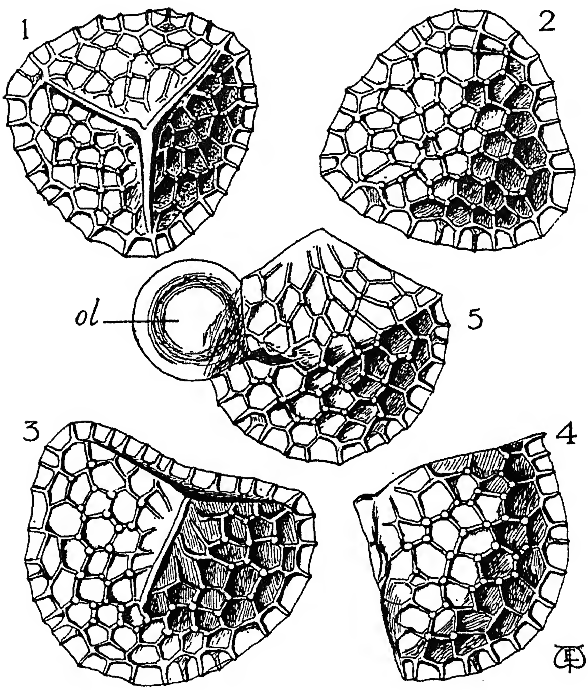

FIG. 44.—Spores of *Lycopodium clavatum*, L.

(1) Spore seen from above to show the apex. (2) Base of a spore. (3) Spore partly turned over. (4) Side view of a spore. (5) A spore burst by pressure upon the cover-glass and a globule of oil, *ol*, emerging from the split. All × 1200.

lycopodium has been mixed with a definite proportion of a second substance, one can find immediately how much of this second substance has been examined microscopically in admixture with the counted lycopodium spores. If the second substance contains any characteristic countable

particles, such as starch, hairs, pollen grains, stone cells, etc., one can quickly calculate the number of such characteristic particles per milligramme which are present in the substance. In this way one obtains a standard figure which represents any such pure material.

The Suspending Agent. For carrying out this work, a suspending fluid is necessary, and experiments with a large number of different liquids have made it evident that the most satisfactory suspending agents are mucilage of tragacanth, olive oil, castor oil, and mixtures of olive and castor oils.

By using olive oil, castor oil, and mixtures of the two, one can obtain a fluid of suitable viscosity at any ordinary temperatures. In cool weather, olive oil alone is sufficient; but in warmer weather castor oil may be added in such proportion as to produce a liquid of suitable viscosity.

When oil is not admissible as the suspending agent, mucilage of tragacanth, either alone or mixed with glycerin, should be used. The mucilage of tragacanth is prepared by mixing 1·25 gm. of powdered gum tragacanth with 2·5 c.c. of 90 per cent. alcohol and adding 100 c.c. of distilled water as rapidly as possible, and shaking vigorously. It should be prepared some hours before it is needed for use, so as to ensure a thorough swelling of the gum.

Example of a Count of Lycopodium Spores. The following example shows how a count of a weighed amount of lycopodium spores is made: 0·1092 gm. of lycopodium was carefully mixed upon a glass plate with mucilage of tragacanth, which was gradually incorporated with the powder by rubbing them together with a flexible spatula. This was transferred to a weighing-bottle, and further quantities of the mucilage were rubbed with the residue of the mixture on the plate and transferred to the bottle until none of the lycopodium remained. The total weight of the spores and mucilage of tragacanth was found to be 11·5814 gms.

A clean microscope slide and a circular cover-glass were

then carefully weighed, and a drop of the well-shaken suspension was mounted on the slide, after which the weight was again determined. The difference 0·0132 gm. or 13·2 mgms. was the weight of suspension on the slide.

The number of spores in twenty fields of view, in positions selected so as to distribute them equally over the preparation, was then counted. The following were the numbers found: 8, 7, 4, 6, 8, 10, 5, 8, 4, 6, 7, 13, 10, 12, 6, 11, 6, 13, 4, 10, giving a total of 158 spores.

The area of one field was found from its diameter measured by a micrometer scale and was 0·2003 sq. mm., so that twenty fields had an area of $20 \times 0{\cdot}2003 = 4{\cdot}006$ sq. mm. The area of the cover-glass was 298·7 sq. mm., so that the total number of spores under the cover-glass was $158 \times 298{\cdot}7 \div 4{\cdot}006 = 11{,}780$. The weight of lycopodium under the cover-glass was $13{\cdot}2 \times 0{\cdot}1092 \div 11{\cdot}5814$ mgms., and this weight contains 11,780 spores, hence 1·0 mgm. contains $11{,}780 \times 11{\cdot}5814 \div 13{\cdot}2 \times 0{\cdot}1092 = 94{,}640$ spores.

The mean of 26 such determinations was 93,000 spores. By calculation from the linear dimensions of the spores the number 95,000 was obtained. The mean value is 94,000 spores per mgm.

Choice of the Fields to be Counted. The twenty fields counted were selected by using a mechanical stage having graduated movements to and fro and right and left, at right angles to one another. The centre of the cover-glass was first adjusted so as to come directly under the front lens of a 4 mm. ($\frac{1}{6}$ in.) objective, the position of the right-hand corner of the slide was read off on the graduated scales, and the slide was then moved by the mechanical stage so as to bring the points indicated on the accompanying diagram (Fig. 45) successively under the lens.

Having determined the number of lycopodium spores per mgm., the characterization of any other suitable material by counting the number of characteristic elements per mgm. becomes a very simple matter. For example,

the number of starch grains per mgm. of maize starch dry at 100° C. has been found as follows (Wallis, *Journ. Roy. Micro. Soc.*, 1920, p. 175) :—

Determination of the Number of Starch Grains per Mgm. of Maize Starch. The number of starch grains

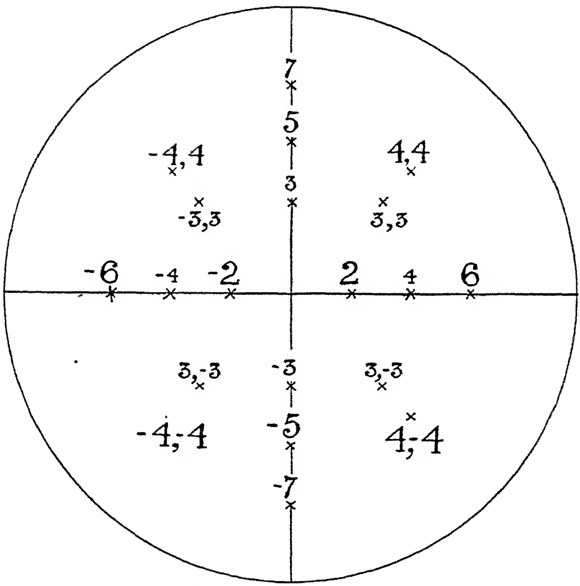

Fig. 45.—Diagram to show the Positions of 20 Fields, selected for Counting.

The position of each field is indicated by figures, giving the distance in millimetres from the central point. If 10 fields only are to be counted, those marked by the larger figures should be chosen.

per mgm. of starch dry at 100° C. can be used to characterize a starch. The figure was found by the following method in the case of a sample of commercial maize starch : 0·2 gm. of lycopodium was mixed with 0·1 gm. of maize starch and about 20 c.c. of olive oil. Four slides were prepared by mounting drops of the suspension, and the

counts of lycopodium spores and maize starch grains were as follows :—

Ten selected fields on the first slide gave

Lycopodium spores 15, 21, 9, 14, 12, 16, 12, 16, 13, 14=142
Maize starch grains. 60, 66, 59, 71, 59, 58, 78, 74, 77, 80=682

Giving 480 maize starch grains for 100 lycopodium spores.

Ten selected fields on the other three slides gave 477, 436, and 410 starch grains respectively, or an average for the four slides of 450 grains for every 100 lycopodium spores. Hence there are $450 \times 94{,}000 \div 100$ starch grains for every mgm. of lycopodium, and since this weight of lycopodium was mixed with 0·5 mgm. of starch, there are $2 \times 450 \times 94{,}000 \div 100 = 846{,}000$ starch grains per mgm. of air-dry starch. Allowing for 13·4 per cent. of moisture, there are $846{,}000 \times 100 \div 86{\cdot}6 = 977{,}000$ grains per mgm. of maize starch dry at 100° C.

In counting the starch grains one counts all particles that are distinctly recognizable as such ; all minute specks having no definite size or form are omitted.

Determination of Maize Starch added to Wheat Flour. By a similar process, the proportion of maize starch added to ordinary wheat flour can be easily determined.

For this purpose one first prepares a 50 per cent. mixture of the two substances, and counts the number of definitely recognizable maize starch grains per milligramme. This figure will necessarily be smaller than that deduced from the number of grains per milligramme of pure maize starch, because certain of the starch grains will be indistinguishable from wheat starch, and are unavoidably omitted from the counts.

For example 0·1000 gm. of lycopodium was mixed with 0·1322 gm. of a 50 per cent. mixture of wheat flour and maize starch, and suspended in olive oil. Ten fields from a preparation made by mounting a drop of the suspension gave the following counts :—

Lycopodium spores	6, 5, 9, 9, 9, 9, 6, 7, 6, 6= 72
Maize starch grains	30, 26, 39, 51, 26, 51, 35, 49, 27, 30=364

Giving 506 maize starch grains for 100 lycopodium spores.

Counts of ten fields from a second slide gave the following numbers :—

Lycopodium spores	10, 7, 7, 16, 9, 10, 5, 10, 6, 4= 84
Maize starch grains	45, 39, 40, 41, 26, 51, 41, 42, 42, 48=415

Giving 494 maize starch grains for 100 lycopodium spores.

These two sets of counts are in close agreement, and give as an average 500 maize starch grains for 100 lycopodium spores, which is equivalent to 470,000 starch grains for 94,000 lycopodium spores, or one milligramme of lycopodium. Since each milligramme of lycopodium was mixed with 1·322 milligrammes of the 50 per cent. mixture, this weight of the mixture, corresponding to 0·66 milligramme of maize starch, contains 470,000 maize starch grains, giving a standard figure of 711,000 grains per milligramme of air-dry maize starch when counted in admixture with wheat flour.

The sample to be analysed was next treated in a similar way by mixing together 0·1032 gm. of lycopodium and 0·1574 gm. of the flour, and suspending them in a mixture of olive and castor oils. Counts of ten fields on the first of two slides gave 104 lycopodium spores to 383 starch grains, and on the second 117 spores to 391 starch grains, making a total of 221 lycopodium spores to 774 starch grains.

Calculation. The calculation is then made as follows :—

The proportions of spores and flour present were 1·032 mgm. to 1·574 mgm., and 1·032 mgm. of lycopodium contains $1{\cdot}032 \times 94{,}000 = 97{,}000$ spores. Hence 1·574 mgm. of the flour contains $774 \times 97{,}000 \div 221 = 339{,}700$ grains of maize starch, which corresponds to $339{,}700 \div 711{,}000 = 0{\cdot}4777$ mgm. of maize starch. Hence the percentage of maize starch in the flour was $100 \times 0{\cdot}4777 \div 1{\cdot}574 = 30{\cdot}3$

per cent. The actual amount present in the adulterated flour was 28·6 per cent.

This result can be made still more accurate by now preparing a standard mixture containing 30 per cent. of maize starch and obtaining a fresh standard count for maize starch in the same way as was done with the 50 per cent. standard mixture.

Mixing the Powders and Preparing the Suspensions. The standard powders must be very carefully mixed, so that a small amount may accurately represent the whole. This can be satisfactorily accomplished by a thorough trituration of the ingredients in a mortar.

Great care must also be taken, when preparing a suspension, to ensure an intimate mixing of the lycopodium and the powder to be examined with the oil or mucilage. Lycopodium spores are remarkably strong, and are very resistant to pressure, so that with suitable apparatus they can be very thoroughly incorporated with the other materials in the suspension. The best way to do this is to mix the weighed quantities of the powders on a glass or porcelain plate by means of a flexible spatula; a little of the suspending medium is added, and the whole worked up into a thin paste, which can be transferred from the plate to a stoppered cylindrical weighing bottle or to a corked tube. The residue on the plate is similarly mixed with a further quantity of medium and transferred to the bottle, and the operation is repeated until the whole of the powder has been removed. The suspension is made up to such a volume as to give about 10 to 20 lycopodium spores in each field of view, when a drop is mounted and examined with a $\frac{1}{6}$ in. objective. The total volume will generally be about 20 c.c.

A drop is taken from the suspension by shaking it vigorously and quickly removing a drop on the end of a thin glass rod. The size of the drop should be such as to just fill up the space under the cover-glass, which is applied in making the mount to be counted.

Quantitative Criteria of Purity. Another useful application of this quantitative method is for the determination of the purity of substances, such as insect powder and powdered cousso. Both these substances consist largely of powdered flowers, and the number of pollen grains present affords an index of the nature and quality of the drug from which the powder was prepared.

Cousso should consist of the pistillate panicles of *Brayera anthelmintica*, Kunth. (N.O. Rosaceæ), and should therefore contain very few pollen grains, only those which have lodged among the floral whorls or are adherent to the stigmas being legitimately present. Staminate flowers are frequently admixed with inferior specimens of the drug, and their presence is revealed by counting the pollen grains per milligramme of the powder. A. Meyer (*Archiv. der Pharm.*, 1908, 246, p. 523) has shown that this number should not exceed 200, so that if a greater number is found the presence of staminate flowers may be inferred.

Preparation of Crude Fibre for Counting. The microscopical appearance of lycopodium spores is unaffected by boiling them with dilute acids and alkalis (see Wallis, *Pharm. Journ.*, July 26, 1919, p. 76), so that when mixed with a powder from which a crude fibre is subsequently to be prepared, the spores appear unaltered in the fibre.

This enables the microscopist to mix lycopodium in a definite proportion with a powdered drug or spice, and to prepare a crude fibre from the mixture with the purpose of concentrating certain characteristic elements or of clearing the preparation sufficiently to render all the countable structures sharply defined and easy of identification. The advantage of the method is that one can immediately ascertain the weight of powder which yielded the crude fibre, in which the characteristic elements have been counted. This weight is found from the proportions in which the two powders were mixed, and from the weight of spores counted, which is calculated from the fact that 94,000 spores weigh one milligramme.

Count of Pollen Grains in Insect Powder. For example, in the examination of insect flowers, one would clear the preparation and concentrate the number of pollen grains by the following procedure :—

Take 1 gm. of the insect powder and mix it with 0·05 gm. of lycopodium. Boil the mixed powders in a porcelain dish for thirty seconds with 50 c.c. of 10 per cent. nitric acid, and filter at the pump through a piece of moistened Horrockses' longcloth, M2, stretched over a Buchner funnel. Wash the residue on the strainer with 100 c.c. of boiling water, and remove it by stretching the cloth tightly, like the membrane of a drum, over the rim of a watch-glass, and scraping it off with a spatula. Return the damp mixture to the porcelain dish, and boil for thirty seconds with 50 c.c. of 2·5 per cent. aqueous caustic soda, filter, and wash at the pump as before.

By the use of a spatula and glass plate, gradually mix the crude fibre so obtained with mucilage of tragacanth until the volume is about 20 c.c. Shake well in a stoppered bottle, and mount a drop for counting with the microscope.

Now count the pollen grains in a strip across a diameter of the cover-glass having a width equal to the diameter of the field of view in the microscope. In a particular experiment this was found to be 71 pollen grains. Next count the number of lycopodium spores in twenty fields evenly distributed along the same diameter ; this was found to be 100, giving an average of five spores per field of view. Measure the diameter of the cover-glass and of the field of view ; these figures were 19 mm. and 0·53 mm. respectively, so that there were 45·8 fields of view in the whole diameter, and, therefore, $5 \times 45{\cdot}8 = 229$ spores.

The calculation is completed as follows : There were 71 pollen grains for every 229 lycopodium spores, and hence $71 \times 94{,}000 \div 229 = 29{,}100$ pollen grains for every milligramme of lycopodium (= 94,000 spores). Hence for 0·05 (one twentieth) mgm. of lycopodium there were $29{,}100 \div 20 = 1450$ pollen grains; and, since 0·05 mgm. of

lycopodium was mixed with every milligramme of insect powder, there were 1,450 pollen grains per milligramme of insect powder.

Lehmann and Trottner (*Revist. Pharm.*, 1917) have shown that insect powder prepared from flower-buds of *Chrysanthemum cinerariæfolium*, Vis. (N.O. Compositæ), contains 2,000 pollen grains per milligramme, and that from partly expanded flowers between 1,000 and 2,000 pollen grains per milligramme. They suggest that any sample containing less than 500 pollen grains per milligramme should be rejected as of inferior quality. The sample examined would, therefore, be one of good quality, so far as this test enables one to judge.

These examples serve to show the various ways in which the lycopodium method can be employed as a quantitative process for the examination of different types of material, and as further research results in the establishment of standards for other substances, the range of application will be gradually extended. The results so obtained show an accuracy quite equal to that of many well-known chemical quantitative processes, and they can be used with confidence in drawing conclusions as to the composition of mixtures or the purity of crude drugs.

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